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BIOLOGICAL METHODS OF SLUDGE DEWATERING

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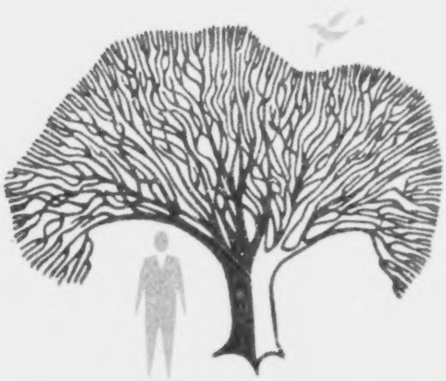

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27 Abstract

Improved dewatering of sewage sludge has been accomplished for the first time by biological means; namely, through the agency of the enzyme lysozyme acting under controlled conditions. Improved dewatering was measured by a 4 to 8-fold increase in filtration rate, which, if extrapolated to plant scale operations, should result, in a given time period, in a 2 to 3-fold increase in solids yield, thus achieving a significant cost saving. A number of other exo- and endo-enzymes were also tested for their effects on dewatering, with uncertain results. Several microorganisms were isolated that showed lysis around their colonies when they were grown on agar plates containing sludge. Attempts were made to enhance this lytic property through the isolation of ultraviolet resistant mutants that exhibited the phenomenon to an increased degree. Selection of matrix-solubilizing organisms was attempted by means of enrichment culture procedures that used an alkaline extract of sludge as the selective medium, which allows the removal of filter-clogging materials that would otherwise obscure the positive action of sludge modifying enzymes. Of major significance is the demonstration that the relative mild conditions of a biological process permit attacking the sludge dewatering process at its source - the matrix material. (Mechalas - Envirogenics Co.)

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BIOLOGICAL METHODS OF SLUDGE DEWATERING

by

Envirogenics Company
A Division of
Aerojet-General Corporation
El Monte, California 91734

for the
WATER QUALITY OFFICE
ENVIRONMENTAL PROTECTION AGENCY

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ABSTRACT

A significant increase in the filtration rate of waste activated sludge has been accomplished by the action of a biological enzyme lysozyme. Improved dewatering was measured by a 4- to 8-fold increase in filtration rate, which, if extrapolated to plant scale operations, should result, in a given time period, in a 2- to 3-fold increase in solids yield. A number of other exo and endo-enzymes were also tested for their effects on dewatering without finding one that produced a consistent improvement in filterability.

The extracellular environment of the sludge flocs, the matrix, contains gelatinous capsular polymers and soluble amino acids but relatively small quantities of proteins and proteolytic enzymes.

Several microorganisms were isolated that showed lysis around their colonies when they were grown on agar plates containing sludge. Attemps were made to enhance this lytic property through the isolation of ultraviolet resistant mutants that exhibited the phenomenon to an increased degree.

Selection of matrix-solubilizing organisms was attempted by means of enrichment culture procedures that used an alkaline extract of sludge as the selective medium.

Of major significance is the demonstration that the relative mild conditions of a biological process permit attacking the sludge dewatering process at its source- the matrix material.

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SECTION I

CONCLUSIONS

The objective of the program, which was to demonstrate that the water retaining properties of waste secondary sludge can be biologically altered, has been achieved.

As a result of these studies there is now a focal point for an applied effort to develop a practical plant scale process. From the information developed in the program, a series of conclusions were drawn as regards the sludge environment and the factors involved in biological sludge dewatering:

1. Treatment of secondary sludge with the enzyme lysozyme dramatically improves filterability.

2. Antibiotics, such as streptomycin, may enhance lysozyme action under given conditions.

3. Anaerobic conditions are essential for lysozyme to alter sludge filterability.

4. The sludge environment is oxygen limited even under aeration.

5. The theory that poly-β-hydroxybutyric acid (PHBA) is the material responsible for floc formation could not be proved. The use of PHBA depolymerase does not affect either floc formation or sludge dewatering.

6. Floc formation and PHBA synthesis may not be causally related.

7. The polymer of the matrix is not an intracellular material released into the medium through the mechanism of cell lysis.

8. The presence of the matrix material and the formation of flocs in activated sludge in the absence of any evidence of cell lysis signifies that the matrix material must be extracellular in origin.

9. A number of species of microorganisms have been isolated that display lytic properties toward sludge when plated on a sludge-containing agar medium.

SECTION II

RECOMMENDATIONS

The finding from this program that an enzyme can bring about faborable changes in sludge filterability provides an essential impetus to the development of a biological dewatering process. For the first time there is direct evidence that a controlled modification of sludge can be brought about through purely biological means. In order to take fullest advantage of the opportunities afforded by these findings, additional information is required before a practical treatment plant application can be developed.

The following is recommended:

1. Undertake a program to determine whether lysozyme acts directly on the floccule matrix or if it serves to release matrix-depolymerizing enzymes from the floccule organisms.
2. Determine the optimal conditions for lysozyme activity with regards to time, temperature, enzyme concentration, etc.
3. Investigate the use of alternative agents with activities similar to lysozyme, including pretreatment of the matrix in order to increase its enzyme susceptibility.
4. Evaluate the effects of sludge solids concentration on:
    a. The effectiveness of lysozyme
    b. The solids yield from processing equipment
    c. The cake moisture
5. Compare production alternatives for economically utilizing the enzyme process in a treatment plant.
6. Develop the requirements for an engineering evaluation of the enzyme process.

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SECTION III

INTRODUCTION

The purification of water in sewage treatment plants is effected by the growth of microorganisms at the expense of organic compounds dissolved in the raw sewage. Although many different organisms can be isolated from sewage cultures, the bulk of the population is thought to belong to the Pseudomonadales, a group of strictly aerobic bacteria to which Zoogloea is probably related. (Dias and Bhat, 1964; Praksam and Dondero, 1967; Stanier, et al., 1966)

In the course of growth, the bacteria in sewage form flocculant aggregates (flocs) that settle quite rapidly out of suspension after the sewage, depleted of soluble organic matter, has been transferred to settling basins. The compacted mass of sedimented bacteria is called secondary sludge; for simplicity it is referred to as sludge throughout this report. The sludge generally occupies about 5% of the total volume of liquid in the settling basin. Although this quantity is proportionately low, the actual volume of sludge produced by large treatment plants is very great. The rapid and inexpensive disposal of sludge therefore becomes a matter of central importance in the treatment of sewage.

The basic problem in disposing of sludge is that of removal of water from it. A variety of chemical and mechanical methods for concentrating the bacteria and draining off the extracellular water have been devised on largely empirical grounds (Fair and Geyer, 1958; Williams, et al., 1970). None of these is very efficient, in the sense that water removal is slow and incomplete (Burd, 1968).

It is of interest that Burd, (1968) the author of this latter reference, which is probably the most complete available survey of sludge handling and disposal, makes only a four line reference to bacteria under a heading devoted to "Sludge Conditioning and Dewatering - Unusual Processes." Microbiologists may well find amusing the suggestion that microorganisms are considered to be "unusual," more especially since microbial activities are foremost in any consideration of sewage treatment.

The primary purpose of this program was to improve the dewatering rate of waste secondary sludge by means of microbial action. More specifically, a search was to be made for bacteria or fungi that have the ability to attack enzymatically the water-retaining matrix material of the sludge floccules. The widely demonstrated enzymatic versatility of bacteria and fungi offered every reason to anticipate the success of this program. One has only to reflect that waste sludges do not accumulate indefinitely in nature to realize that something is ultimately disposing of them. In that microbes are the final arbiters of the fate of all other known organic substances, microbes being implicated in final sludge disposal is a reasonable assumption. A priori, therefore, sludge-attacking enzymes must exist in nature.

The first phase of this overall study was directed toward the treatment of secondary sludge with an assortment of enzymes specific for known bacterial polymers. In that the polymer(s) comprising the sludge matrix are undoubtedly produced by the bacteria embedded in the floccules, the chemical composition of the polymers of the floc could be considered to be similar to the extracellular polymers elaborated by other bacteria. For reasons indicated below, the initial emphasis was placed on producing and evaluating the depolymerase for poly-β-hydroxybutyric acid, a polymer claimed to be responsible for the physico-chemical properties of sludge.

Enzymes in general have optimal activities over a well-defined range of environmental conditions and deviations on either side of these optima can markedly affect activity. Additionally, in that enzymes are proteins, they are subject to inactivation and destruction by the same forces that denature other proteins. Thus, there is the requirement that prior to the use of any enzyme the environment in which it is to act must be defined.

In this program, sludge from the Pomona, California, sewage treatment plant was regarded as a typical secondary sludge and its physiological environment characterized. In the second half of the study, Whittier Narrows secondary sludge was also studied. The conditions necessary for optimal enzyme activity may themselves have an affect on filtration rates and identification of what changes are brought about independent of enzyme treatment becomes important. The effect of pH and of the chemicals used to buffer the reaction mixtures had to be defined in that enzymes differ in their responses to these parameters. Once the conditions that affect the active life time of enzymes in a sludge mixture had been characterized, and the non-enzyme factors that influence dewatering rates had been identified, the work progressed to the final stage of exposing sludge to various selected enzymes and determining their effect of filterability.

The principal objective of this program was to increase the rate of filtration of secondary sludge. The measure of the effectiveness in achieving this was evaluated by determining the specific resistance of a sludge before and after treatment. A ten-fold decrease in specific resistance means that the filterability has increased by a factor of ten. An improvement of this magnitude is considered highly significant since this reflects a doubling or tripling of the solids yield to be expected from a vacuum filter in a given time.

The dewatering of sludge by filtration has received considerable attention, partly because filtration could theoretically eliminate nearly all of the extracellular water, and partly because the rate of filtration correlates will with the compactability of sludge by other methods (Coackley (1958); Cummins (1950)). Several types of continuous vacuum filters are currently being used in sewage treatment plants. In the course of filtration, however, the bacterial filter cake becomes gummy and extremely resistant to the further flow of water, and accordingly this method has little practical value at present.

The difficulty of filtering sludge can be partially attributed to the presence of ionizable functional groups associated with the flocs, for the filtration rate increases dramatically after sludge is either acidified or incubated with trivalent cations, such as Fe^{+++} or Al^{+++} (Busch (1966); Busch & Stumm (1968)). This meagre evidence throws little light on the biochemical properties of sludge, and more extensive information is required before we can hope to manipulate the floc in such a way that sludge can be dewatered rapidly to completion.

In that the floc matrix is formed during the growth of the sewage organisms, it may be assumed to be under some sort of metabolic control. Recent work by Crabtree, et al. (1966) demonstrated a relationship in Zoogloea ramigera, a major bacterial inhabitant of sewage sludge, between floc formation and production of the intracellular food reserve poly-β-hydroxybutyric acid (PHBA). These workers speculated that because this polymer has physical properties similar to sludge and since it accumulates coincident with cell aggregation, it could be responsible for formation of the floc.

Interest in developing a biological method of sludge dewatering originated from the speculation of Crabtree, et al. (1965) (1966), that flocculation was caused by intercellular bridges of poly-β-hydroxybutyric acid. Such bridges could be formed either by the extrusion of β-hydroxybutyryl strands from the cells, or by free polymer granules liberated by cellular autolysis. It was reasoned that if this suggestion were correct, it would be possible to change the filtration rate of sludge by treatment with poly-β-hydroxybutyric acid depolymerase.

Of the known enzymes that hydrolyze the PHBA polymer, the extracellular depolymerase of Pseudomonas lemoignei was chosen as the most suitable for experimental purposes (Delafield (1964)). It is a true exoenzyme that accounts for much of the extracellular protein produced during exponential growth of the bacterium, and its purification is accordingly unnecessary; it hydrolyzes all known forms of poly-β-hydroxybutyric acid, but not other esters; it is stable, highly active, and easy to assay by turbidimetric methods. A drawback to using this enzyme is its inability to hydrolyze polymer granules whose surfaces are occluded by absorbtion of various basic proteins, such as might well be present in sludge. This disadvantage, however, can be offset by treating protein-coated polymer with a self-digesting proteolytic enzyme, such as trypsin, before adding the depolymerase.

Bacteria are known to store other polymeric materials in their cytoplasm and these are normally released into the surrounding medium upon cell autolysis. Because the physical properties of many of those materials resemble those of sludge, the test program included enzymes known to degrade polymers of bacterial origin. The objective of these experiments was not a complete dissolution of the floc matrix so that cell dispersion would occur, but rather a controlled or partial depolymerization so that the physico-chemical properties of the matrix would be altered. Such changes should be reflected in altered sludge filterability.

Enzymes are proteins, and thus there is a strong possibility that the addition of enzymes purposely to sludge would result in their hydrolysis by preexisting proteolytic enzymes produced by floc bacteria. Moreover, intracellular enzymes that may be effective against floc matrix could also be inactivated by such enzymatic action when the intracellular enzymes are released into the suspending medium. It is highly desirable to selectively control this activity and prevent such hydrolysis from taking place.

By inhibiting metabolic activity, protein synthesis may be reduced to the point where new proteolytic enzymes are no longer produced in the sludge, thereby permitting added enzymes to survive. Another approach would be to alter selectively the cell membrane permeability and allow only certain enzymes to leak out. Some antibiotics are known whose activity involves the inhibition of protein synthesis and the modification of membrane permeability; streptomycin is such an antibiotic (Davis, et al., 1968). Other similar biologically active compounds include the antibiotics penicillin (affects cell wall) and chloramphenicol (inhibits protein synthesis), and the enzyme lysozyme (affects cell permeability). Many other examples exist. In addition, control of the cells' environment, such as by the creation of anaerobic conditions, could produce similar effects.

The second phase of these dewatering studies centered around the attempted isolation, from likely sources, of bacteria or fungi that possess the ability to degrade enzymatically the floc matrix. In order to facilitate isolation, enrichment culture techniques were used (Aaronson, 1970). Essentially, this methodology involves the use of a basal culture medium, complete in all nutrients except one. This latter nutrient is supplied by the compound of interest. Thus only organisms capable of utilizing this compound will be able to grow and survive. For example, in the present study, it was generally conceded that the floc matrix material was chemically most likely to be carbohydrate composed in turn of one or more polysaccharides. Therefore, in order to isolate a floc matrix-metabolizing microorganism by the enrichment technique, chemically extracted floc matrix material was added as the only carbohydrate source to a basal medium containing nitrogen and various salts. This completed medium was then inoculated with sources known to contain large numbers of different microbial species, e.g., soil, lake mud, and animal feces. If an organism is present in the inoculum that can grow in the medium, i.e. that can utilize the extracted floc material, it may be selected through repeated transfer in the same medium. In this case, if the organism can attack extracted floc matrix material, it may well be able to attack the native matrix *in situ* in the flocs.

If such a floc-attacking microbe were isolated, one could envisage a sludge treatment facility where this organism could be grown to large numbers in a small subsidiary tank. This culture could then be periodically fed into the main body of sludge at such a rate as to bring about a predetermined degree of sludge dewatering.

Another approach involved the isolation on autoclaved sludge agar of bacteria and fungi from a variety of sources. Sludge agar was readily prepared by adding 1.5% agar to sludge and autoclaving for 10 min. The criterion used for isolation was the appearance of zones of clearing around well separated colonies in the otherwise opaque sludge agar. Such "sludge lysers" were tested for their ability to attack both native sludge and chemically extracted sludge matrix material.

An added parameter to the above was the attempted isolation from the original "sludge lysers" of mutant lytic organisms showing enhanced lysis after ultra-violet irradiation. As discussed later in this report, a number of organisms were exposed to ultra-violet light followed by a variety of conditions of incubation, dilution, etc., in an effort to obtain a microorganism capable of dissolving the floc matrix.

Studies involving the supplementation of sludge were also conducted. The reasoning followed here was that sludge may be regarded as an incomplete nutrient medium that could be made complete if the right nutrients or growth factors were added. This could cause the rejuvenation of micro-organisms in the sludge, possibly with metabolic utilization of the sludge matrix. Accordingly, a number of nitrogen and carbon sources that may have been preferentally removed during the sewage treatment process were added to sludge to reestablish it as a growth medium.

Toward the end of this project a major change in philosophy toward dewatering experiments took place. Slower filtration of a treated sludge sample, when compared with the filtration of normal sludge, had heretofore been taken to indicate that the treatment was not efficient in dewatering sludge. The conclusion was developed that these data could be regarded in a very different way. Instead of slow filtration resulting from the absence of positive action of a given treatment, perhaps slow filtration resulted because the treatment *had* been effective, leading to the freeing of bacteria from floccules and the clogging of the filtration apparatus. Such filter clogging by freely existing bacteria is a tedious phenomenon long known to bacteriologists. An additional possibility is that viscous by-products result from successful enzyme action and alter the filtration of the suspending liquor. In other words, effective treatment, i.e., deflocculation or dewatering, results in either the freeing of bacteria from the flocs or in the formation of viscous products and the consequent masking of successful sludge modification.

The use of enzymes and antibiotics was an obvious extension of the program discussed to this point. The complex ecology of sludge suggested the requirement for inhibiting at least a portion of the metabolic activity of its microbial population. This approach ultimately led to a combination of environmental conditions and mixtures of lysozyme and streptomycin that dramatically altered the filtration rate of sludge.

SECTION IV

SLUDGE: CHARACTERIZATION AND PARAMETRIC DESCRIPTION

EXPERIMENTAL DESIGN

The experimental program was divided into three main areas of investigation. One of these was to determine if the sludge environment could be manipulated in such a way that deflocculation would result. Theoretically this would be the easiest and most desirable method, if it proved to be successful.

In that sludge is a complex organic material that is eventually degraded under natural conditions, it is probable that microorganisms are responsible for this decomposition. Therefore an effort was initiated to isolate microorganisms that possessed the ability to metabolize the polymers that comprise the sludge matrix.

Since enzymes are the arbiters of microbial activity, it follows from the above that enzymes are the logical mechanisms by which organisms attack sludge. Because many enzymes are known that are able to decompose polymeric materials of microbial origin, a major endeavor was undertaken to examine these known enzymes for their effects on sludge filtration.

In order to determine the effectiveness of the various experimental treatments, a method that measured the filtration rates of the samples was adopted. This method simulated actual plant operation and presented the data in such a way that an engineering evaluation was possible.

MATERIALS AND METHODS

Preparation of Poly-β-hydroxybutyric Acid

The asporogenic strain KM of Bacillus megaterium was grown in the basal medium of Williamson and Wilkinson (1958) supplemented with 0.1 M sodium acetate and 0.05 M glucose as sources of carbon. The bacteria were cultivated at 30°C in 16 liters of the medium contained in carboys, with aeration supplied through spargers. A good growth yield (2 g dry weight/liter) was not obtained unless the bacteria were serially subcultured in small quantities of the medium for several days before inoculating the carboys. The bacteria were harvested by centrifugation in the early stationary phase of growth, and their intracellular polymer was purified by the method of Williamson and Wilkinson (1958), but using commercial "Clorox" at pH 9.8 instead of the alkaline hypochlorite prescribed by these workers. The final yield of polymer was about 25% of the dry weight of the bacteria.

Preparation of Enzymes

Poly-β-hydroxybutyric Acid Depolymerase

Pseudomonas lemoignei was grown while being shaken at 30°C in the basal medium of Delafield, et al. (1965), supplemented with 0.8% disodium succinate as the source of carbon. The culture was centrifuged when the bacteria were in the late exponential phase of growth and the supernatant solution was used as the source of depolymerase without further purification. This crude enzyme was stored before use at 0°C, without loss of activity, throughout the duration of this work.

Crystalline Enzymes

Crystalline enzymes treated were: α-amylase, β-amylase, cellulase, chitinase, DNAase, lipase, lysozyme, peptidase, pronase, RNAase, and trypsin. All except trypsin were dissolved in water at a final concentration of 5 mg protein/ml. Trypsin was dissolved at the same concentration in 0.001 N HCl. The enzymes were stored at 0°C, and were used within three weeks to avoid possible loss of activity associated with prolonged storage in the cold.

Determination of Enzymatic Activity

Poly-β-hydroxybutyric Acid Depolymerase

Enzyme activity was estimated from the rate of decrease in turbidity of a standard suspension of polymer granules. Changes in turbidity were followed in a Klett-Sommerson photoelectric colorimeter with a number 66 filter. Because colorimetric readings are not directly proportional to the concentration of particles in suspension, the instrument was calibrated by plotting the actual Klett readings as a function of polymer before digestion. Turbidity is a function of particle size as well as number and thus, this relationship changes during digestion of the polymer granules. The change, however, introduces only slight error, and no attempt to correct for it was made in this work (Delafield, 1964).

The standard suspension of polymer was made by briefly homogenizing dry polymer in distilled water, and then subjecting the suspension to ultrasonic vibration in a Bronwill Biosonik III for five minutes. The suspension contained 6.5 mg polymer/ml (75 μequivalent of β-hydroxybutyrate/ml).

One unit of depolymerase is defined as that quantity of enzyme causing the hydrolysis of poly-β-hydroxybutyric acid at an initial rate of one μequivalent of hydroxybutyric acid residues solubilized per minute at 25° in a mixture containing 1.5 μeq polymer, 50 mols of Tris HCl buffer at pH 8.0, and 1 μmol $MgCl_2$ per ml. The reaction is started by addition of the polymer, and the enzyme must be below a concentration that saturates the surface of the polymer granules. The crude enzyme used in this work had an activity of 0.8 units/ml and saturated the polymer at 0.27 units. These values are in close agreement with those previously published in a detailed study of the depolymerase. (Delafield, 1964). The actual kinetics of the reaction are complex so that the detailed studies should be consulted before attempting to work with the enzyme (Delafield, 1964; McLaren, 1969).

Proteolytic Enzymes

The activities of trypsin, pronase, peptidase, and unidentified protease(s) in sludge were measured by following the release of free amino acids from Bovine Serum Albumin. Reaction mixtures contained 4.0 ml 0.1 M Tris HCl buffer at pH 7.6, 0.5 ml 0.1 M $CaCl_2$, 2.0 ml Bovine Serum Albumin at 5 mg/ml, and water or enzyme to give a final volume of 10 ml. Enzyme was added last to start the reaction. At intervals thereafter, 0.4 ml aliquots of the reaction mixtures were combined with 0.8 ml 10% trichloroacetic acid (TCA). After incubation for 30 minutes at 23°, precipitated protein was removed by centrifugation and the supernatant liquid was analyzed for amino acids by the methods described below. In the case of sludge, solids were centrifuged before precipitating protein in the supernatant liquid. The kinetics of hydrolysis were not examined in detail, and no attempt was made to define units of activity.

Other Enzymes

Assay procedures for the other enzymes used in this study were not developed. All of these enzymes were fresh commercial preparations and examination of their kinetics was considered unnecessary unless they could first be shown to have a demonstrable effect on the filterability of sludge.

Supernatant Replacement

The conclusion was developed that processes showing promise for altering matrix material might be overlooked as a consequence of the screening procedures. Thus, a new approach to dewatering experiments was utilized. Experiments were now conducted in two parts. First, treated samples, including controls, were filtered in the same manner as previously and the results recorded, and second, similar samples were then centrifuged at low speed (1000 rpm) for 5 min. Under these conditions, the sludge centrifuged into a soft pellet (which could be resuspended readily) whereas any free bacteria or viscous by-products remained in the supernatant, being unaffected by this relatively low gravitational force acting for such a short space of time. The supernatant fluids were then removed by means of a Pasteur pipette or an aspirator, and the pellet brought back to the original volume with fresh sludge supernatant fluid. These samples were filtered in the usual way and compared with the non-centrifuged samples. This procedure often led to dramatic increases in the rate of filtration of treated samples. Since control sludge was processed in the same manner in each test, this experimental approach to dewatering would appear to be valid.

Sedimentation

The rate of sedimentation was expressed as the volume occupied by solid matter after one liter of sludge was allowed to settle for one hour in a one-liter cylinder. Attempts to scale this method down to volumes compatible with enzymological work were not successful. Enzymatically treated samples of sludge were allowed to settle in 10 ml (11 mm I. D.) cylinders, and the rate of sedimentation was compared with that of appropriate control suspensions. However, the samples, whether treated with enzyme or not, tended to break up into buoyant clumps, and estimation of their total volume in the cylinders was not possible. Data from these experiments were accordingly meaningless, and are omitted from the report.

Sludge Filterability

A modification of the Buchner funnel technique (Fair and Geyer, 1958) was used to measure sludge filterability as specific filtration resistance (r) as described in the Appendix. Evaluation of changes in filterability were based on comparing plots of the filtration rate as θ/V^2, where θ = time in seconds and V = filtrate volume in ml. In general, these plots could be divided into three successive phases: (1) a brief initial phase with a low slope (rapid filtration), (2) a prolonged secondary phase that lasted until about 80% of the water in the sludge was removed, and (3) a final phase with a steep slope (slow filtration). The second phase was used to calculate the slope, b.

This method has two serious drawbacks. First, in many cases the experimental points in the second phase do not form an unambiguous linear sequence, and accordingly estimation of the slope with any degree of precision is difficult. The variability associated with this lack of precision can be seen in the experiments, and the filtration rates reported have an estimated standard deviation of ± 20%. Second, the formula for calculating specific filtration resistance implies that the filtration rate is inversely proportional to the solids content of the sludge. This implication is not strictly correct, for when samples of sludge are concentrated by various methods to the same solids content they can show very pronounced variations in filtration rate. The reason for this behavior is unknown. Observations were made, however, that when sludge is concentrated by any method and then serially diluted, the subsequent filtration rates show strict inverse proportionality to the solids content of each sample. In view of this observation, the sludge was treated in the following standard fashion. Fresh sludge was centrifuged at 23° in a DeLaVal Centrifuge, and the bacterial mass was resuspended to about 5% dry weight in the clear supernatant liquid by stirring with a spatula until a homogenous suspension was obtained. This suspension is referred to as concentrated sludge. In the enzymatic work, aliquots of concentrated sludge were incubated with enzymes or with a volume of water equivalent to the enzyme mixture. After suitable periods of time, the aliquots were diluted with equal volumes of buffer and were filtered. In order to cencel out any subtle variables that might contribute to erratic filterability, the filtration rates of the enzymatically treated samples were compared with those of the appropriate controls. The variations actually observed were therefore attributable to the graphical inaccuracy previously described.

MICROSCOPIC OBSERVATIONS

In order to be able to generalize from the enzyme experiments reported here, the secondary sludge selected for study must be "typical" of most activated sludge processes. The microflora, chemical composition, and physical properties should be defined.

Fresh sludge was taken for experimentation from the Old Treatment Plant at Pomona, California, or the Whittier Narrows plant near El Monte, California. These plants use the Activated Sludge Process. In practice the process operates as a continuous culture, but for the sake of simplicity it can be considered as a batch culture in order to set forth clearly the intervals of time of interest here.

The plant's primary effluent is aerated in the presence of 0.2% sludge for 6 hours. Aeration is then discontinued, and the resulting floc is allowed to settle for 3 hours, following which the clear supernatant liquid is decanted over a wier, which retains the settled sludge. Roughly 3% of the sludge in the aeration tank is removed every three hours, and this is pumped to a trunk line leading to a Los Angeles County facility for further treatment and disposal. The sludge used in this work was drawn from the trunk lines. By the time the sludge reached the Envirogenics laboratory it had been without active aeration for roughly 4 hours (this includes time in the secondary settling tank). The sludge (10 liters in a 15-liter carboy) was immediately re-aerated through a sparger in the laboratory at an air flow of 5 ℓ/minute.

Observation by phase-contrast microscopy showed the flocs to be similar to those described by other workers. Owing to the thickness of the floc particles, individual bacteria are difficult to see, but when they can be visualized, as shown in Figure 1, they are slightly separated and appear to be embedded within some sort of a matrix, for the individual cells are without Brownian motion. When stained and examined by light-microscopy, the bacteria in the floc are universally Gram negative, an observation which is consistent with the notion that they are predominantly Pseudomonadales. The sludges contain fewer protozoa than might be expected, though many of the flocs enclose large numbers of apparently encysted ciliates. A wide variety of protozoa are also present in sludge, including Vorticella sp., Lionotus sp., Euplotes sp., Peranema sp., Amoeba sp., and Pelomyxa sp. Rotifiers are also abundant. Spirilla are occasionally encountered. Furthermore, the interstitial space between the flocs is invariably teeming with free bacteria that were at first thought to be Spirilla. Samples of this sewage were submitted to Dr. Hans Veldkamp of the University of Groningen, who was visiting at UCLA at the time, and he identified these spirillum-like organisms as Spirochaeta plicatilis (Figures 2 and 3). This organism was discovered

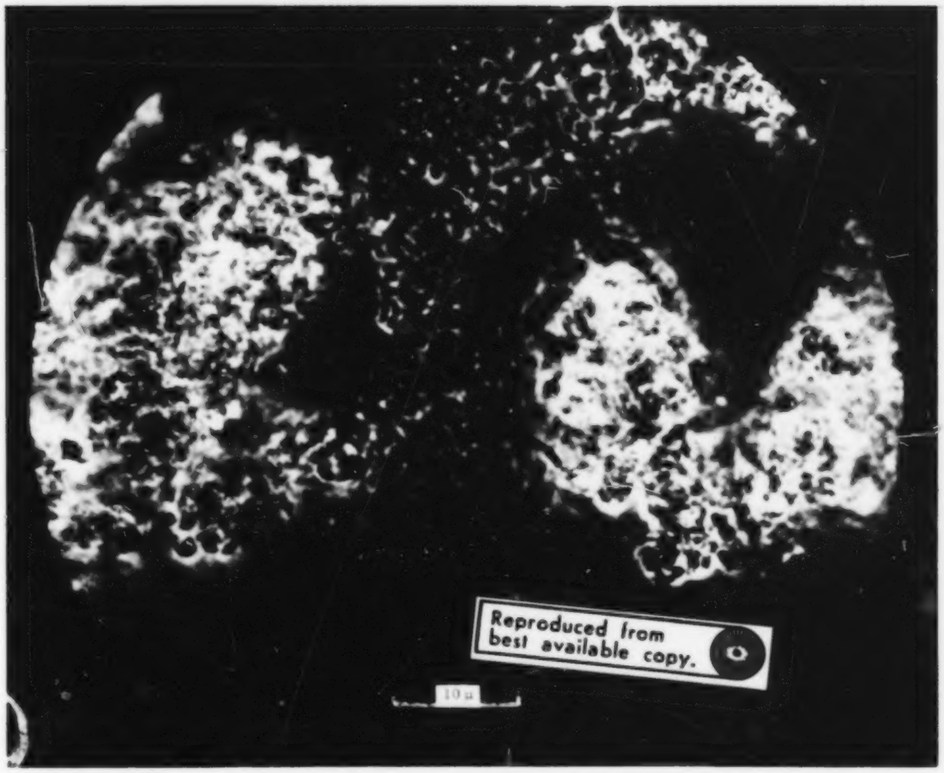

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Figure 1. Phase Contrast View of Activated Sludge Floc. Individual cells are randomly oriented and appear to be imbedded in a matrix.

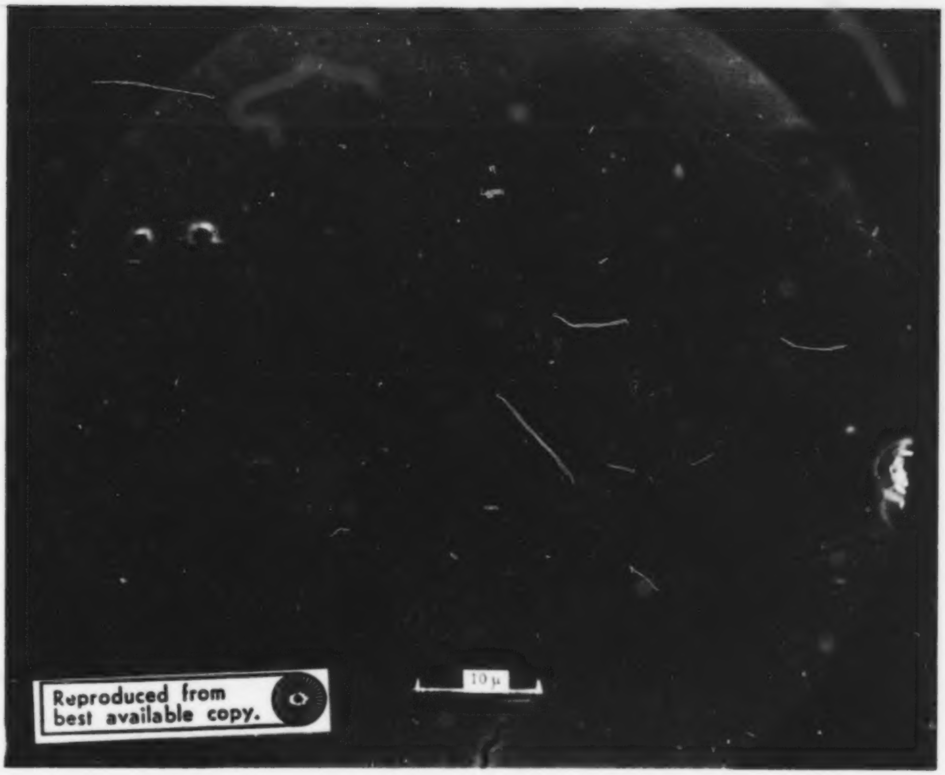

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Figure 2. Phase Contrast View of Spirochaeta Plicatilis in Activated Sludge. Secondary coils visible in organism above the reference line.

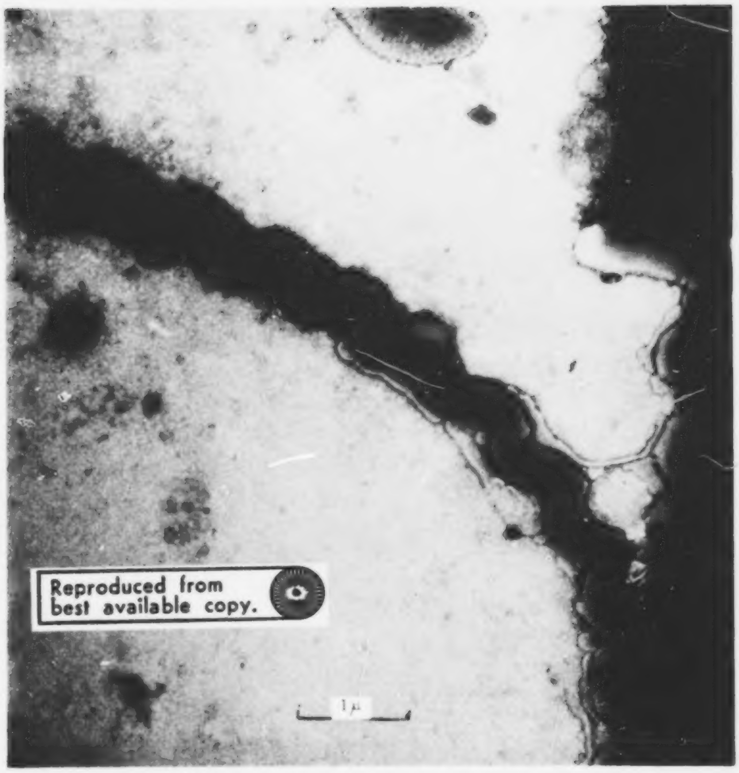

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Figure 3. Spirochaeta Plicatilis as Viewed Through the Electron Microscope. Coils are tightly wound around a well-defined axial filament.

in soil by Dyar (1947) in 1936, but it appears to be relatively unknown, and has not been reported as a common inhabitant of sludge. Examination of a sample of return sludge obtained from the Los Angeles Hyperion treatment plant also showed the presence of large numbers of these organisms and they apparently are a more common inhabitant of sludge than has been thought.

Since there are at present no adequate criteria for comparing different sludges, the foregoing qualitative observations permit no definite conclusion about the normality of the sludges used in these studies. The possibility exists that the unknown factors that lead to growth of S. plicatilis also lead to other changes in the bacterial population of the sludge, but if such changes do in fact exist, they appear to have no gross effect on the normality of sludge, as judged by its physical characteristics. The notion that the physico-chemical properties of sludge tend to remain fairly constant in the face of dramatic population changes is supported by the experiments of other workers (Cowie and McClure (1959)). Examination of this idea in greater detail would have been interesting, but such a study was beyond the scope of this work.

PHYSICO-CHEMICAL CHARACTERISTICS

The physical characteristics of Pomona sludge are similar to those reported for other sludges. As shown in Table 1, the specific resistance, sedimentation rate, and content of carbohydrate and poly-β-hydroxybutyrate of Pomona sludge fall within normal limits (Coackley (1958); Crabtree, et al, (1966)). The actual values of these four parameters fluctuate slightly from day to day, but there is no apparent correlation between the fluctuations.

Table 2 presents data on the percent solids and sedimentation rates for Whittier Narrows (WN) sludge. It will be noted from Tables 1 and 2 that whereas the two lower range values for the per cent solids of the Pomona and the Whittier Narrows sludges are virtually the same, their two upper range values are quite disparate, being considerably higher for the Whittier Narrows sludge. This reflects the much higher average value for per cent solids found in routine sampling of the WN sludge.

Studies on Floc Disaggregation

Many industries have problems similar to those posed by sludge dewatering in that they are faced with removing colloidal suspensions from their products. One of the solutions to this problem is to add gelling agents that either form a structure that collapses and traps the colloidal material or that neutralize surface charges, permitting agglomeration. These procedures are common in the food and beverage industries; e.g., beer, coffee, wine, etc.

Because many such gels are produced biologically, it was important to determine if materials of this type could influence the dewatering properties of sludge. Similarly, an additional solution to the problem is to

TABLE 1

CHARACTERISTICS OF POMONA SLUDGE

Date	% Solids	Specific Filtration Resistance (sec^2/g)	Settled Vol.[‡] (% of 1 Liter) (60 minutes)	PHBA[+] (% of Solids)	Anthrone[+*] (% of Solids)
12/11/68	0.71	1.1×10^9	-	5.1	13.0
12/30/68	0.71	9.2×10^8	-	5.0	-
1/13/69	0.62	1.1×10^9	85.0	5.2	-
1/14/69	0.68	8.9×10^8	84.5	6.4	6.0
1/15/69	0.66	9.0×10^8	82.5	7.9	6.3
1/16/69	0.66	9.1×10^8	81.0	7.4	7.3
1/17/69	0.71	9.1×10^8	84.0	8.2	9.3
2/11/69	0.60	1.3×10^9	42.0	4.5	6.2
2/13/69	0.70	1.3×10^9	61.0	7.2	6.6
2/21/69	0.72	9.5×10^8	75.0	7.4	8.6
2/24/69	0.87	1.2×10^9	83.0	5.5	9.4
2/26/69	0.87	1.3×10^9	81.0	8.4	9.1
2/28/69	0.73	1.3×10^9	77.0	-	7.0
Average	0.66	1.1×10^9	-	6.0	8.2
Range	0.62-0.87	8.9×10^8-1.3×10^9	-	5.0-8.4	6.2-13.0

[‡] 1 liter graduated cylinder, 60 mm in diameter.

[+] PHBA = Poly-β-hydroxybutyric acid.

[*] Materials giving a positive color for carbohydrates as shown by the Anthrone test. See Appendix.

TABLE 2

SOLIDS CONTENT AND SETTLING RATES OF WHITTIER NARROWS SLUDGE

Date	% Solids	Settled Vol. (% of 1 Liter in 60 min.)*
8/18/70	0.75	89.8
9/15/70	0.72	91.7
9/23/70	0.73	94.0
10/13/70	0.97	95.0
10/20/70	0.78	92.0
10/26/70	0.91	95.0
11/5/70	0.68	87.0
12/17/70	0.92	92.0
12/29/70	0.75	95.0
2/1/71	0.79	87.5
2/15/71	0.93	89.5
3/10/71	0.94	94.5
3/25/71	0.88	73.0
4/1/71	0.64	39.0
4/20/71	0.77	94.5
5/11/71	0.75	96.5
5/19/71	0.86	95.0
Average	0.81	
Range	0.64-0.97	

*1 liter graduated cylinder, 60 mm in diameter.

bring about the dispersion of the floc by agents that either affect the amount of cross-linking between polymer strands - urea - or that result in alteration of surface charges of the molecules - heat.

An increasingly common practice in waste treatment plants is the aerobic digestion of waste secondary sludge as a disposal method. This results in a dense sludge with enhanced dewatering properties and a supernatant that does not impose an additional organic load on the plant. Such an environment must stimulate the selection of microorganisms that possess enzymes capable of altering the physical chemical make-up of the floc matrix. This aspect and those mentioned above involving urea and heat were explored in the experimental program and are described in Appendix 2, along with the results.

EXTRACTION AND CHARACTERIZATION OF MATRIX

In order to proceed with this program, it was important to have some idea of the chemical composition of the floc material. This information would allow selection of candidate enzymes that are known to attack similar classes of compounds. In addition, microorganisms that have the ability to degrade complex molecules could be isolated if the matrix material were offered as a growth substrate.

Several different microbial polymers have been reported and characterized in the literature. Methods for extracting these polymers in a purified form have been developed and these have been used to advantage in the present study.

Alkaline Extraction

Since it was assumed that the floc polymer is made up totally, or partially, of polysaccharides, alkaline extraction, which is used in isolation of polysaccharides, was one of the methods investigated.

A number of modifications of the basic technique described below were tried in order to extract the maximum amount of material making up the matrix of activated sludge floccules. Essentially, these modifications involved varying the pH, the time of the extraction, and the amount of the organic solvent used to precipitate the extracted material. For example, sodium hydroxide (0.1 - 1.0 N) was added to a sludge concentrated by centrifugation and the pH of the mixture maintained at different pH's ranging from 8.5 to 12.5. The length of time allowed for extraction at alkaline pH's in different experiments ranged from 15 minutes to 18 hr. Methanol was added to the extracted material in various amounts from one-to six times the volume of the extracted material. Extraction was conducted both in the cold in ice baths using ice-cold reagents and also at room temperature employing ambient temperature reagents. Sodium chloride was added as well as sodium hydroxide in several of the procedures in an effort to increase the yield of the extracted polymer.

In the end the basic technique, which has been applied to sludge, in amounts up to 15 liters, consists of treating concentrated sludge at room temperature with 1 N NaOH (final pH 10.0), and 0.5-1 M NaCl for about an hour, with constant stirring. The sludge is then centrifuged and the brown supernatant fluid decanted. The extracted material is then precipitated with 3 volumes of absolute methanol, with stirring. The gray gelatinous precipitate that results is then collected by decanting and centrifugation and resuspended in deionized water. The precipitates did not go into solution, but formed suspensoids.

Dry weight determinations have shown that approximately 15% of the sludge material is extracted by this procedure. That more polymer could not be removed by this technique is taken as an index of the chemical complexity of the matrix material.

Hot Water Extraction

In that various polysaccharides are soluble in hot water, aqueous extractions of sludge at different temperatures were tried. Centrifuged sludge was resuspended in 100 ml of hot deionized water at the temperature desired for the particular extraction, i.e., 50, 60, 70, 80, and 100°C, and placed in a hot water bath at that same temperature for a period of 30 min. At the end of this time the reaction vessel was immersed in an ice bath to stop the reaction. Centrifugation in the cold yielded supernatant that were treated with 3 volumes of cold absolute methanol. Any precipitates that formed were collected and resuspended in small amounts of deionized water.

It was of interest that precipitates formed only from 70° and 80°C samples and that they formed suspensoids in water.

Acetone Extraction

One hundred ml of cold deionized water was added to centrifuged sludge, the mixture made into a paste, and placed in a blender that had been wrapped in a plastic bag containing ice in order to keep the contents of the blender cold. The paste was blended at the maximum speed of the machine for 30 min., at which time the temperature of the paste had risen to 51°C, in spite of the ice collar. After centrifugation, the supernatant fluid was treated with 3 volumes of cold acetone and the resultant gray, gelatinous precipitate was resuspended in a minimal amount of deionized water. This material was saved for further characterization.

Extraction by Sonication

One liter of fresh Whittier Narrows sludge was centrifuged, the sediment resuspended in 500 ml of deionized water and 100 ml aliquots subjected to sonication in an ice bath by means of a Bronwill Bisosonik III sonicator. The progress of the sonication was monitored by observing preparations at intervals under the phase contrast microscope. Five minutes of operation at 80% capacity of the Biosonik were sufficient to markedly break up the floccules, although it also caused some bacteria to become ghosted and eliminated any evidence of motility. The suspension was centrifuged and the solids discarded. The recovered supernatant represented 16% of the sludge-starting solids on a dry weight basis. To a portion of the solution four volumes of methanol were added and the resultant precipitate was separated and resuspended in deionized water. As will be described, this extract was included in subsequent analytical tests.

Chloral Hydrate Extraction

The general method of Bourne and Weigel (1965) for the extraction of bacterial polysaccharides was followed. One hundred ml of WN sludge was centrifuged, the pellet was added to 100 ml of a 33% solution of chloral hydrate in water, and stirred repeatedly for one hour.

Examination in the dark phase microscope revealed an immersion refractometry effect because of the high concentration of chloral hydrate; i.e., the bacteria appeared as if they were being viewed by bright phase microscopy and they seemed to be flating in the absence of a matrix material that evidently had the same refractive index as the chloral hydrate. The sludge itself became lighter brown in color and looked curdled. When the fluid portion was passed through a No. 1 Whatman filter paper, a yellow filtrate was obtained. A gel formed when the filtrate was streamed into 250 ml of acetone. This was collected by centrifugation and dry weight determination of it indicated that 11% of the floccule had been extracted by this procedure.

At the conclusion of this phase of the program, four different samples were available that had been extracted from sludge by methods selective for polysaccharides. It was intended to choose one or more of them as the nutrient substrate in the enrichment culture technique. However, it was possible that nucleic acids were simultaneously extracted from the sludge organisms along with the polysaccharides. Contamination of the samples with large amounts of nucleic acids would confuse the results of the enrichment studies. Thus it was important to determine if nucleic acids were present in the extracts. The evidence for ruling out the presence of large amounts of nucleic acids in these samples is presented in Appendix B, along with the test procedures that were used.

In that time did not permit extensive analysis of the extracts obtained by various means, the alkaline extraction procedure was chosen in order to procure sufficient material to perform enrichment culture procedures.

ENRICHMENT FOR MATRIX-DEGRADING MICROORGANISMS

An interesting technique common to microbiology is the use of enrichment cultures to select for organisms capable of attacking specific substrates. This is based on the principle that given a heterogenous population of microorganisms that are placed in a basal synthetic medium whose only source of carbon and energy is the compound of interest, only those organisms possessing the requisite enzymes to attack it will survive and proliferate. By serially transferring the organisms to fresh medium as they reach peak growths, the organisms containing the required enzymes will eventually populate the culture.

Alkaline extract material, usually at 10% (v/v), was incorporated as the potential carbohydrate source into a basal medium used for the cultivation of Pseudomonas. This consisted of the following ingredients:

Solution 1:

K_2HPO_4	2.6 g
KH_2PO_4	2.1 g
NH_4Cl	1.0 g
$MgSO_4 \cdot 7H_2O$	0.5 g
Deionized water	1 liter

Solution 2:

Ferric ammonium citrate	1.0 g
$CaCl_2$	0.1 g
Deionized H_2O	100 ml

This medium was sterilized by autoclaving for 10 min at 120°C and 15 lb/in.^{2}. After cooling, 0.5 ml of Solution 2 was added per 100 ml of Solution 1.

Inoculated flasks containing the basal medium (90 ml) and the alkaline extract (10 ml) were incubated either at 30° in a shaker water bath or at room temperature (approximately 22°C) on a gyrotary shaker.

The following inocula were used as possible sources of microbes able to attack extracted matrix material, and, by extension, possibly native floccular matrix material in situ: a number of field soils, animal dung (horse, rabbit, goat, guinea pig), lake bottom mud, sludge from stones taken from the trickling filter at the Azusa, California, installation, water from the lagoon at the Azusa plant, soil from beside the Azusa trickling filter, and African violet potting soil.

The enrichment technique consisted of transferring, 1 to 3 days after inoculation, a large volume (5 to 10 ml) from the inoculum-containing flask (passage 1) to another flask of the same basal medium + alkaline extract material (passage 2). Sequential passages were made in the same medium. The flasks were examined microscopically for the presence of predominant bacteria that appeared to utilize the alkaline extract as a carbohydrate source. The contents of the flasks were also streaked out periodically on a sludge agar medium in order to detect colonies of microbes that possessed enzymes that could attack the sludge; this was indicated by clearing zones around the colonies. Matrix digesting organisms were not observed.

"Lytic" organisms, as defined by clear "halos" or zones of dissolved sludge around the microbial colony, were obtained from a variety of sources and inoculated into test tubes containing Pseudomonas medium + alkaline extract. Similarly, plates containing this medium plus 1.5% agar to solidify it were also inoculated. These organisms produced luxuriant growth in the tubes and on the plates without reducing the turbid medium that results from the presence of the alkaline extract. In other words, these "lytic" organisms were also not using the extracted material. A pure culture of Zoogloea ramigera grew well on, or in the above media, but, again, without reducing the opacity of the alkaline extract. Fresh sludge spread on the above solid medium gave rise to many colonies, none of which produced any evidence of clearing of the extract material. Many other variations have been followed in attempts to isolate floc attacking microorganisms, among them being the attempt to isolate such an organism(s) directly from sludge. A small amount of sludge was inoculated into the Pseudomonas basal medium containing added alkaline extract material. Even after three passages in this medium, the matrix material appeared to be unused and sludge agar plates spread at various times from the liquid cultures gave no evidence of lysis.

Attempts to Establish Competitive Populations in Sludge

A number of attempts have been made to establish growth of pure cultures of microorganisms in or on fresh, native sludge. These experiments have mostly involved the inoculation of "lytic" organisms into vessels containing aerated liquid sludge, with the view that the organisms might become established and exercise their "lytic" property against flocs in their native environment. Other experiments have tried to obtain growth of "lytic" organisms on sludge agar plates in which the sludge has either not been autoclaved or has been sonicated to break up the flocs and then incorporated into cooled agar. In no instance were the inoculated organisms able to compete successfully with the native sludge population. One attempt employed a very large number of bacteria as an inoculum as indicated below.

"Lytic" organism Stock No. SL-27 was inoculated from a stock culture into a flask of sterile nutrient broth that was incubated at room temperature on a gyrotary shaker. Good growth occurred in 18 hours and this was used to heavily inoculate 29 autoclaved sludge agar plates, which were then left overnight at room temperature.

Excellent growth resulted, after which the organisms were washed off each plate by means of tap water and a bent glass rod, and then pooled. The suspension of bacteria (about 125 ml) was centrifuged at high speed and the resulting pellet, containing a tremendous number of active bacteria, was scraped out of the centrifuge tubes and used to inoculate 500 ml of fresh sludge, which was subsequently aerated by means of a compressed air line. A second, uninoculated, flask was similary aerated as a control.

Microscopic examination at 22 hours did not reveal any difference between the contents of the two flasks; i. e., the SL-27 inoculum could not be detected. There was thus no reason to continue and the experiment was terminated.

Attempts were made to grow three known "lytic" organisms in fresh sludge that had been sonicated for 15 minutes to break up the floccules. This procedure was carried out in the cold to prevent heating of the sludge by the disruptive energy of the sonication; the pH remained at 6. 8.

The sonicated sludge, still containing many viable cells, was inoculated with a massive culture of "lytic" organisms. Although the inocula could not be detected microscopically at 19 hr or 48 hr, tests on the respective flasks were made to see if the sedimentation rate of the inoculated sludges had been grossly affected by the presence of the added organisms. It was found, in fact, that the sonicated sludge, including the control, did not settle out appreciably, even after standing in 1 liter graduated cylinders for a day.

The haloing effect observed on autoclaved sludge agar provided a means for visualzing changes in floc composition. Accordingly, this medium with and without nutrient additives was used as a selective medium to screen for sludge lysing organisms. These experiments, while interesting, did not immediately contribute to major alterations of the floc material and the data are presented in the appendix are experiments describing attempts to develop mutants possessing enhanced lytic properties. These efforts centered on exposing selected sludge lysers to ultra-violet irradiation. One attempt to work with molds is also described in that section of Appendix B.

SECTION V

SLUDGE MODIFICATION

FILTRATION CHARACTERISTICS OF SLUDGE UNDER DIFFERENT CONDITIONS

Filtration of Untreated Sludge

Inasmuch as different enzymes have maximal activity at different pH values, and since they furthermore may solubilize acidic or basic components in the course of digesting sludge, the behavior of sludge with respect to pH was examined. The effect of Mg^{++}, which is required as a cofactor by α-amylase and poly-β-hydroxy-butyric acid depolymerase, was also examined.

Titration of the supernatant liquid from centrifuged sludge shows no detectable buffering capacity (Figure 4). When 100 ml of the solution was acidified to pH 1.85 with roughly 1.9 moles of HCl, 1.7 moles of NaOH were required to titrate the solution to pH 10, as measured with the glass electrode. The titration curve is that of a strong acid, and reflects neutralization of the HCl initially added. About 0.2 mole of the added acid was not recovered in the titration; this loss can be attributed to the evolution of CO_2 at pH 1.85, and it corresponds to a carbonate content in the sludge of roughly 200 mg/l as Na_2CO_3. In a similar experiment with whole sludge, 100 ml of sludge was acidified to pH 1.90 with 2.7 moles of HCl; 3.2 moles of NaOH were required to titrate this suspension to pH 10. As shown in Figure 5, whole sludge has a weak buffering capacity with a pH of roughly 7. In fresh sludge containing about 0.5% solids there are approximately 0.015 meq titrable acidity per ml.

The titration curve of whole sludge resembles that of protein. It could be attributed either to amino acids, to protein, to other polymeric materials in the sludge, to ionizable groups on the bacterial surface, or to any combination of these factors. Whatever the case, the materials would have to be largely insoluble, since they do not appear in significant amounts in the supernatant liquid. These results indicate that for experimental purposes the pH of the sludge should be poised by buffers at a concentration of 0.1 - 0.2 M, depending on the concentration of solids in the material.

Effects of pH Buffers and Salts

The effect of pH on the filtration rate of sludge was examined next. Filterability was determined by measuring the filtrate volume accumulated versus time using a vacuum filtration apparatus. A filtration resistance θ/V^2 was calculated based on the above measurements. This is directly related to specific resistance, r, which is a measure of filterability. Details of this procedure are provided in materials and methods and in Appendix A.

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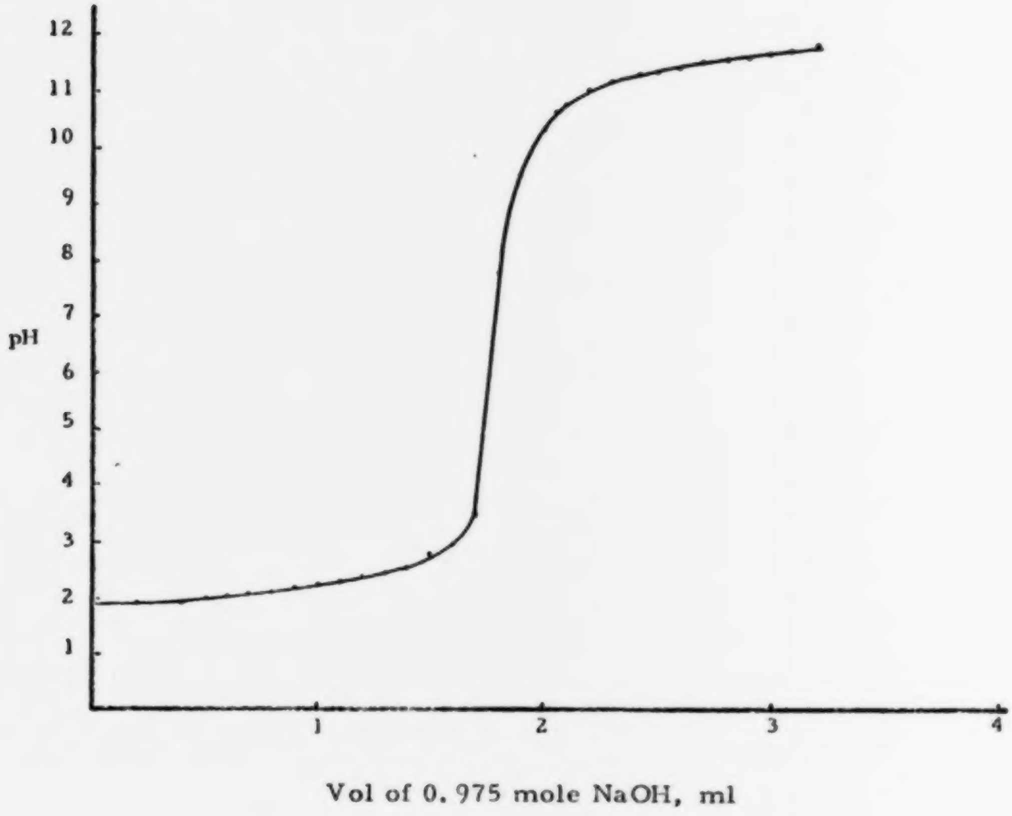

Figure 4. Buffering Activity of Sludge Supernatant 100 ml of the supernatant liquid from centrifuged sludge was titrated with NaOH.

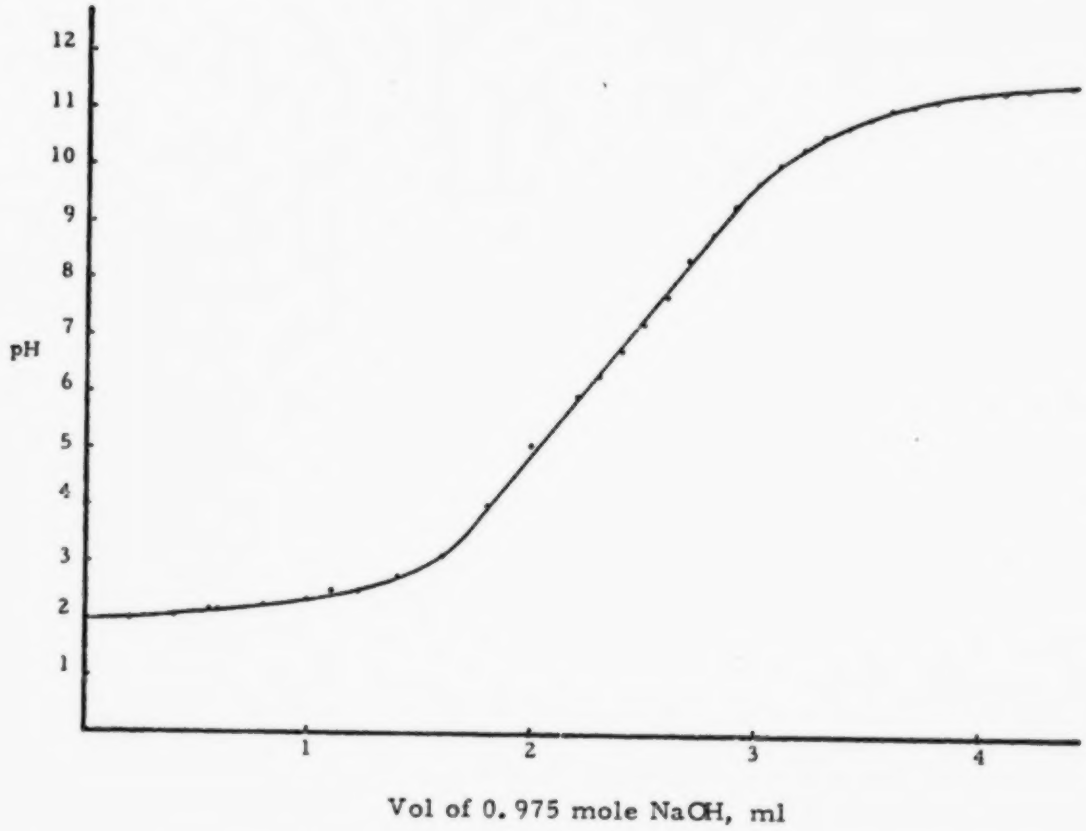

Figure 5. Buffering Activity of Sludge 100 ml of whole sludge was titrated with NaOH.

Since the physico-chemical properties of sludge are complex, the behavior of sludge was studied first in which the pH was adjusted to different levels by adding HCl or NaOH in the absence of exogenous buffers. As shown in Table 3, the rate of filtration was very rapid at pH 3, but grew slower at more acid or alkaline levels. There is a possibility that at pH values greater than 3 the rate of filtration is inversely proportional to pH. In contrast to these results, the rate of sedimentation appears to be independent of pH, although a kinetic study of sedimentation might well prove this supposition to be false. Finally, it may be noted that the variations in this experiment cannot be attributed to changes in the viscosity of the supernatant liquid, as might *a priori* be supposed, for the viscosity was basically the same in all the filtrates.

A similar experiment, in which the pH was poised by different buffers, was now performed, and the results are shown in Table 4. In general, the observations described in the previous experiment are again apparent. The presence of citrate buffer, however, leads to a striking reduction in the rate of filtration. Thus, at pH 6.3 - 6.5, the rate of filtration is about four times slower in citrate buffer than in phosphate buffer. This observation could be attributed to the formation of a complex between citrate, which is a chelating agent, and some metal(s) whose presence in the sludge is essential for rapid filtration. If so, the inverse proportionality between filtration rate and pH in the presence of citrate would be attributable to an increasing concentration of carboxylate ions as the pH is raised.

Effects of $MgCl_2$ on Sludge

If the above interpretation is correct, it should be possible to partially or completely reverse the inhibitory effect of citrate by means of metals exogenously added to the sludge. To examine this notion, the rate of filtration was compared in samples of fresh sludge. These samples were adjusted to pH 6 with either HCl or citrate buffer and incubated in the presence or absence of $MgCl_2$, the cofactor of interest for the depolymerizing enzymes, before their filtration rates were tested. The concentration of several metals in the filtrates was also measured in order to discover any possible correlation between removal of metals from the sludge matrix and filtration rate.

As shown in Table 5, relatively small amounts of magnesium, calcium, and iron occur in unbuffered filtrates. Furthermore, their concentration is unchanged when the sludge is preincubated with $MgCl_2$ in concentrations up to 100 mM. It may be noted that the amount of magnesium in the filtrates of samples 3 and 4 is roughly equal to the quantity of magnesium added, plus the quantity that would have appeared without added magnesium. This observation suggests that there is no significant equilibrium between free and bound magnesium in the sludge. Finally, the filtration rate of unbuffered sludge appears to be the same whether or not magnesium is added.

TABLE 3

Effect of pH on the filterability of sewage sludge

26 ml of fresh sludge were adjusted to arbitrary pH values with HCl or NaOH, and the volume was made to 30 ml. After incubation for 30 min at 23°C, the sludge (20 ml) was filtered through Whatman #1 filter paper in a 22 mm diameter funnel with a sintered glass tube. Initial pH of sludge = 7.4. Solids content = 0.67/100 ml (= 0.587 % diluted).

pH			Graphical Estimate of b = θ/V^2*	Viscosity of filtrate 25°C centistokes
Initial	At 30 min	Of filtrate		
2.05	2.10	2.15	16.0	.908
2.65	2.90	2.90	6.6	.913
4.00	4.72	4.50	40.0	.949
4.80	5.15	6.00	40.0	.946
5.80	6.05	7.00	44.0	.903
6.60	6.70	7.70	50.0	.902
8.30	8.10	8.30	51.0	.909
9.30	9.10	8.60	57.0	.913

*Method for determining θ/V^2 is described in Appendix. In general, θ/V^2 is directly related to specific resistance, r, which is a measure of filterability.

TABLE 4

Effect of pH on Filterability of Sewage Sludge

26 ml of fresh sludge were adjusted to different pH values with HCl or NaOH, and the volume was made to 30 ml. The filterability of these mixtures was compared with that of similar mixtures prepared with the following buffers: Citric acid-Na-citrate (pH 3, 4, 5, and 6; lactic acid-Na-lactate (pH 3); acetic acid-Na-acetate (pH 4 and 5); Na_2HPO_4-KH_2PO_4 (pH 6 and 7); Tris-HCl (pH 7, 8, and 9). The final concentration of each buffer was 0.05 mole. After incubation for 30 min at 23°, the filtration rates were determined by methods described in the appendix. Initial pH of sludge = 7.4. Diluted solids = 0.59% (column A) or 0.73% (columns B-F).

	Graphical Estimate of θ/V^2					
pH	A No buffer	B Citrate	C Lactate	D Acetate	E Phosphate	F Tris
2	27					
3	11	19	32			
4	68	114		90		
5	68	277		90		
6	75	310			90	
7	85				80	56
8	53					67
9	97					67

In contrast to these observations, the concentrations of all three metals in the filtrate of sludge buffered with 20 mM citrate increase in comparison with the filtrates of the control reaction mixtures. The increase is approximately two-fold for magnesium and calcium, but is twenty-fold for iron. The rate of filtration shows a concomitant drastic reduction. However, when this sludge is incubated with either 20 mM or 100 mM $MgCl_2$, the rate of filtration doubles, whereas the concentration of iron in the filtrate decreases by half. The concentration of calcium is also reduced, though to a lesser extent than iron. The magnesium concentration is again unchanged, as was noted in the precending paragraph.

Effect of Chelating Agents on Sludge

In order to examine the possibility that iron is the functional element in the foregoing experiments, the effect of α,α -dipyridyl and Tiron on the filtration rate of sludge was tested. These compounds form complexes with ferrous and ferric ion, respectively; Tiron will complex other transitional elements as well. The effect of these reagents, as shown in Table 6 is reproducible; when sludge is incubated with dipyridyl, its filtration rate decreases by a factor of 2. A similar effect is not produced by Tiron. The quantity of iron in the filtrate of sludge treated with Tiron is higher than the control, indicating that the concentration of ferric ion may not critically affect the dewatering rate. Further investigation of this might be desirable, for these observations invite the interesting speculation that the filterability of different sludges may vary somewhat according to the concentration of iron in the sewage. The iron may be in the form of ferrous rather than ferric ion, a notion that is compatible with the observation that sludge is anaerobic, or reducing, as will be subsequently discussed.

Effect of Addition of NH_4Cl on Dewatering of Sludge

The ability of sludge to synthesize depolymerizing enzymes when stimulated by a source of ammonia nitrogen (Crabtree, 1966) was tested by measuring changes in dewatering characteristics.

A 10 ml sample of a 2.9% sludge concentrate was diluted with 400 ml of sludge supernatant. This dilution was necessary to facilitate supplying oxygen to the sludge biomass. To this sample was added 4 ml of 5% NH_4Cl and the mixture was incubated for 16 hours (Sample A). After incubation, 50 ml of A were used to treat 100 ml of a 2.9% sludge concentrate (Sample B). As a control, 2.9% sludge concentrate was diluted 1/1 with supernatant. After 1 hour incubation, all samples were vacuum filtered to determine the relative dewatering rates. The θ/V^2 of the dewatering rates are indicated in Table 7. It is apparent that the addition of NH_4Cl directly to the sludge concentrate did not appreciably alter the dewatering rate.

These experiments have yielded some significant information as a prerequisite to interpreting the effect of enzyme treatment on sludge dewatering.

TABLE 5

Effect of $MgCl_2$ on Sludge at pH 6 in the Presence and Absence of Citrate

22.5 ml of fresh sludge were adjusted to pH 6 with either HCl or 0.2 mole pH 6.0 citric acid-citrate buffer. $MgCl_2$ was added, as noted in the table, and the volume of every mixture was then made to 30 ml with H_2O. The final concentration of citrate buffer was 0.02 mole; the final concentration of $MgCl_2$ was either 0.02 mole or 0.1 mole. After incubating the mixtures for 30 min at 23°C, their filtration rates were determined. Initial pH of sludge = 7.6. Solids content = 0.5% (w/v) undiluted.

Reaction Mixtures	pH poised with	pH			Mg^{++} added mM	Metals in Filtrate			θ/V^2 Estimated Graphically	Viscosity 23°C, Centistokes[(a)]
		Initial	At 30 min	In filtrate		Mg^{++*} mM	Ca^{++*} mN	Fe^{++*} mM		
1	--	7.6	7.6	7.9	0	3.8	1.3	.0054	16	0.878
2	HCl	6.0	6.3	7.3	0	2.1	2.2	.0054	16	0.896
3	HCl	6.1	6.2	7.3	20	22.0	2.7	.0054	20	0.891
4	HCl	6.0	6.1	7.2	100	101.0	2.9	.0054	28	0.920
5	Citrate	6.0	6.1	6.5	0	4.1	4.5	.0940	84	0.917
6	Citrate	6.0	6.1	6.3	20	26.4	3.5	.0480	52	0.922
7	Citrate	6.0	6.2	7.0	100	106.0	2.5	.0480	46	0.934

(a) The density of Sample No. 1 was 0.9983 g/ml. The density of the other samples can be estimated from this value, ad viscosity of centipoises may be calculated from the formula $\eta_{cp} = \eta_{ch}$ x density.

* Methods used for determining the metals are described in Appendix i.

TABLE 6

Effect of Chelating Reagents on Filtration Rate

Fresh sludge was concentrated by settling to 1.56% (w/v) solids. Reaction mixtures contained 12.0 ml sludge, 6.0 ml 0.1 mole pH 7 phosphate buffer, and (1) 6.0 ml H_2O; (2) 3.0 ml H_2O and 3.0 ml 10 mmole α,α-dipyridyl; (3) 3.0 ml H_2O and 3.0 ml 10 mmole α,α-dipyridyl; (3) 3.0 ml H_2O and 3.0 ml 10 mmole Tiron; or (4) 3.0 ml 10 mmole α,α-dipyridyl and 3.0 ml 10 mmole Tiron. The mixtures were incubated for 30 min at 23°C, and their filtration rates were determined. The concentrations of Mg^{++}, Ca^{++}, and Fe^{++} in the filtrates were also measured.

Reaction Mixtures	Chelating Agents added	θ/V^2	Metals in Filtrate		
			$Mg^{++(a)}$ mM	$Ca^{++(a)}$ mM	$Fe^{++(b)}$ mM
1	none	30	1.37	0.85	0.014
2	α,α-dipyridyl	82	1.37	1.37	0.023
3	Tiron	34	1.00	1.37	0.029
4	α,α-dipyridyl and Tiron	57	0.88	1.33	0.027

(a) Mg^{++} and Ca^{++} were determined by titration with EDTA.

(b) Iron was determined by the penanthrolene method after reduction to Fe^{++} by boiling with acid and hydroxylamine.

TABLE 7

Effect of NH_4Cl on Dewatering Rates

Sample	Reaction Mixture	% Solids	θ/V^2	$\frac{\theta/V^2 \text{ Control}}{\theta/V^2 \text{ Treated}}$
A	10 ml 2.9% sludge, 400 ml supernatant; 4 ml 5% NH_4Cl	0.75	14	-
B	A + 2.9% sludge (1/4)	1.41	96	1.20
C	2.9% sludge supernatant (1/1)	1.48	116	1.00

Enzyme activities are strongly affected by the pH of the environment. In order to insure maximal activity of the enzymes, the sludge will have to be buffered in the range optimal for activity. The titration studies reported in this section indicate that the buffers must be at a concentration of 0.1 - 0.2 mole to overcome the buffering effect of the sludge. The fact that pH in itself affects the dewatering rate stresses the importance of appropriate controls in interpreting results of experiments and the danger that favorable enzyme activity may be masked by an adverse pH requirement.

The presence of $MgCl_2$, a cofactor for many of the enzymes of interest, does not interfere with the filtration rate of sludge.

Free proteins, which would be expected in the event of mass cell lysis, were not detected in the sludge liquor. This implies that if any proteins were released they must have adsorbed to insoluble material. Such proteins could well block sites for enzymatic digestion of various polymeric compounds in the sludge, thus inhibiting the effectiveness of depolymerizing enzymes.

Care should be taken in selecting buffers or other adjuvants to be used in conjunction with enzymes. Removal of unidentified metal(s) from sludge by chelating agents such as citrate reduces the filtration rate.

ALTERATION OF SLUDGE DEWATERING RATES

The inability of cultures of sludge-"lysing" microorganisms to display lysis when inoculated into living sludge presented the possibility that an inhibitory mechanism may be involved. This inhibition could take several forms; for example, competition between different species within the floc, enzyme repression within microbial cells, changes in cell permeability, etc. Additionally, it is possible that the catalytic quantities of enzymes added to the sludge may not be able to diffuse through the matrix material in sufficient concentration to modify dewatering properties. Use of large amounts of enzymes may bring about significant physiological changes in the sludge.

Because certain antibiotics are able, partially or completely, to restrict the growth of bacteria through effects on the cell envelopes, it was decided to observe the changes resulting from the addition of penicillin (which inhibits some Gram-positive bacteria) and streptomycin (which inhibits some Gram-negative bacteria) to activated sludge. It seemed reasonable to speculate that these antibiotics might inhibit the growth of certain of the bacteria in sludge and thereby allow others to increase in number, possibly even promoting bacterial species that might be able to attack sludge matrix material. That is, if matrix-dissolving species are normally present in sludge in small numbers, perhaps the presence of antibiotics, alone, or in combination with enzymes, might permit the enhancement of these species.

Antibiotics

Streptomycin sulfate (final concentration of 10 mg/ml), penicillin G sodium (final concentration of 10,000 units/ml), and streptomycin plus penicillin in the same respective concentrations, were each dissolved in flasks containing 100 ml of fresh WN sludge; 100 ml of sludge was also used untreated as a control. The flasks were incubated at room temperature on a gyrotary shaker and wet mount preparations were than made at intervals and examined in the phase microscope. At 22 hr and at 6 days, filtration tests were made on the above antibiotic-containing sludge; the results are shown in Table 8.

The table indicates that in the amounts used the addition of penicillin, streptomycin, or the two in combination, reduced the filtration rate at both 1 and 6 days after the addition of the antibiotics. However, when the technique of supernatant replacement was employed at 6 days, there was a definite increase in the rate of filtration of the streptomycin sample and a lesser, but noticeable, increase in the penicillin plus streptomycin combination. Presumably this latter positive effect resulted from the presence of the streptomycin, although its effect was apparently reduced one-third owing to the presence of penicillin.

Microscopic observations revealed that in less than 6 hrs after its addition penicillin, alone or together with streptomycin, caused the spirochaetes to become immobile, probably indicating death. In the streptomycin-containing flask, frantically twisting spirochaetes could be seen by 3 hrs after the addition of this antibiotic; spirochaetes still exhibited these violent motions at 22 hr. More extra-floccular, i.e., free, bacteria were seen in all of the antibiotic treated samples, beginning about 3 hrs after their addition, than were obvious in the control sample. On the 3rd day, the fluids of the streptomycin and the streptomycin plus penicillin flasks seemed to have become more viscid; many free bacteria were noticeable in all of the antibiotic-containing flasks at this time but notably in the streptomycin containing sample – this would appear to be reflected in the supernatant replacement experiment. Six days after the addition of the antibiotics, the antibiotic-treated sludge samples contained floccules that had a granular appearance.

Experiments employing streptomycin alone were repeated, in part, as follows:

Streptomycin in the following amounts, 0.1 g, 0.2 g, and 1 g, was added to each of three flasks containing 100 ml of WN sludge. The flasks were sealed with Parafilm and allowed to stand undisturbed for 23 hr at room temperature, at which time the flask contents were shaken in order to disperse the sludge uniformly, and then filtered in the customary manner.

The filtration rates were plotted and compared with those of a normal sludge control that had also been kept at room temperature, sealed and undisturbed. The results showed that the only noticeable effect of

TABLE 8

Dewatering of Penicillin and Streptomycin Treated Sludge

One million units of Penicillin G Sodium and 1 g of Streptomycin Sulfate were each dissolved separately or together in 100 ml of fresh sludge and incubated at room temp. on a gyrotary shaker. The filterability of these mixtures was compared with that of untreated control sludge at times indicated.

Sludge Treatment	θ/V^2	$\frac{\theta/V^2 \text{ Control}}{\theta/V^2 \text{ Treated}}$	Supernatants Replaced θ/V^2	Supernatants Replaced $\frac{\theta/V^2 \text{ Control}}{\theta/V^2 \text{ Treated}}$
At 22 hours				
Control sludge	25.0	1.0		
+ Penicillin	133.3	0.2		
+ Streptomycin	57.1	0.4		
+ Penicillin + Streptomycin	133.3	0.2		
At 6 days				
Control sludge	100.0	1.0	15.7	1.0
+ Penicillin	333.3	0.3	50.0	0.3
+ Streptomycin	400.0	0.25	5.0	3.1
+ Penicillin + Streptomycin	500.0	0.2	14.2	1.1

streptomycin was to slightly decrease the filtration rate at the 1.0 g level of streptomycin. Supernatant replacement did not affect the rate of filtration; the curves were virtually superimposable one upon the other.

Increased Quantities of Enzymes

The enzyme screening previously described made use of catalytic amounts of enzymes. The attempt was then made to increase the substrate available to the enzyme by concentrating the sludge solids. A second approach to the problem of working with a dilute substrate is to increase the enzyme concentration, thereby leaving the substrate in its normal condition. By employing massive doses of enzymes, diffusion barriers would be overcome, a larger proportion of the substrate would be altered, and enzyme activity could be maintained over a longer period of time.

The use of the massive enzyme technique in combination with antibiotics was used in a series of experiments indicated below.

Pectinase: An experiment was carried out in which 1 g and 0.5 g of pectinase were added to separate flasks of fresh Whittier Narrows sludge. One gram of the enzyme was also added to 100 ml of fresh WN supernatant to serve as a pectinase solubility control. Flasks were incubated on a gyrotary shaker at room temperature.

The next day it was patently obvious that pectinase is relatively insoluble in sludge supernatant fluid. Nonetheless, it was decided to filter the samples. The results revealed that the 1 g sample filtered much faster than the normal sludge control, whereas the 0.5 g sample possessed a filtration rate not significantly greater than that of the control. The increased filtration rate of the 1 g sample is considered to be attributable to the pectinase that did not go into solution; i.e., that physical, rather than biological, factors were involved.

Under the phase microscope, both of the pectinase samples appeared viscid and there were many free cells among the normal appearing flocs.

Lysozyme: Lysozyme (or muramidase) is an enzyme whose substrate is found in the cell walls of various species of bacteria.

The effects of lysozyme on filtration rates are shown in Table 9. The highest concentration of lysozyme, viz., 0.5 g, when incubated under aerobic conditions, produced the fastest filtration rate; this was already evident by 2-1/2 hr after it had been added to the sludge, and was much more marked by 22 hr. At 2-1/2 hr, the 0.25 g amount of the enzyme seemed to parallel the effect of the larger amount of lysozyme, but this effectiveness was seemingly lost by 22 hr, since the filtration rate then only equaled that of the control. Lysozyme in the lowest concentration, 0.1 g, reduced the rate of filtration below that

TABLE 9

Dewatering of Lysozyme-Treated Sludge - Shaker Culture

3 x Crystallized Lysozyme (Sigma Chemical Co.) was dissolved directly in 100 ml of fresh sludge and incubated at room temperature on a gyrotary shaker.

Sludge Treatment	θ/V^2	$\frac{\theta/V^2 \text{ Control}}{\theta/V^2 \text{ Treated}}$	Supernatants Replaced	
			θ/V^2	$\frac{\theta/V^2 \text{ Control}}{\theta/V^2 \text{ Treated}}$
At 2.5 Hours				
Control	7.7	1.0		
Lysozyme 0.5 g	5.2	1.5		
Lysozyme 0.25 g	5.9	1.3		
Lysozyme 0.1 g	14.6	0.5		
At 22 Hours				
Control	42.8	1.0	16.7	1.0
Lysozyme 0.5 g	15.8	2.7	7.7	2.2
Lysozyme 0.25 g	42.2	1.0	15.4	1.1
Lysozyme 0.1 g	50.0	0.9	16.7	1.0

of the control. Interestingly, the supernatant replacement experiment did not produce any increased efficiency of filtration.

Examination of wet mount samples in the phase microscope at 22 hr showed a large number of spherical, protoplast-, or spheroplast-like forms in the 0. 5 gm lysozyme sample, and a progressively smaller number in the 0. 25 g and 0. 1 g samples, respectively.

Table 10 indicates the conditions under which lysozyme was retested at 3 concentrations. The main difference between these and previous experiments is that the present ones involved Parafilm sealed flasks left undisturbed at room temperature, thus approaching anaerobiosis, until immediately before the filtration tests. Under these experimental conditions, there was a dramatic increase in the dewatering rates of the lysozyme-treated samples when compared with the control. This indicated a significant alteration of the floc matrix.

For reasons considered in the Discussion section, the dewatering values presented in Table 10 are more extensive than indicated by the figures. Supernatant replacement actually lowered the values slightly.

Enzyme-Antibiotic Combinations

The markedly improved dewatering obtained through the use of lysozyme under controlled environmental conditions (anaerobiosis) and the suggestion of improvements by antibiotics, led to tests of the effectiveness of these agents in combination. Time permitted the initial investigation of only those enzymes considered most likely to exhibit an improved dewatering effect. Most of this work was completed before the requirement for anaerobiosis was appreciated.

A commercial product trademarked Cellulase 36 (Rohm and Haas Co.), which is of microbial origin and which can attack cellulose, pectin, pentosans, and hexosans, was tested for its activity against sludge floccules.

Flasks containing 100 ml each of fresh Whittier Narrows sludge were set up as follows:

1. Control sludge
2. Cellulase 36 (1 g)
3. Cellulase 36 (1 g) + Penicillin (10, 000 units/ml)
4. Cellulase 36 (0. 5 g) + Penicillin (10, 000 units/ml).

Solubility tests indicated that 0. 5 g of Cellulase 36 went into solution in 100 ml fresh sludge supernate but that 1 g of this preparation was not completely soluble.

At 17 hr, there were a relatively large number of free cells in all of the flasks, including the sludge control. Filtration experiments carried out at this time showed that there had been little or no enzymatic activity against the floccules, even after replacement of the supernatant fluids (Table 11).

TABLE 10

Dewatering of Lysozyme-Treated Sludge - Still Culture

Lysozyme was added to flasks containing 100 ml each of sludge, as indicated. Flasks were sealed with Parafilm and left undisturbed at room temperature. Immediately before the filtration tests, the flasks were shaken to give uniform suspensions of sludge and added directly to the filtration apparatus.

			Supernatants Replaced	
Sludge Treatment	θ/V^2	$\frac{\theta/V^2 \text{ Control}}{\theta/V^2 \text{ Treated}}$	θ/V^2	$\frac{\theta/V^2 \text{ Control}}{\theta/V^2 \text{ Treated}}$
At 22 Hours				
Control	12.5	1.0	7.7	1.0
Lysozyme 0.1 g	5.6	2.2	4.3	1.8
Lysozyme 0.2 g	3.6	3.5	3.4	2.3
Lysozyme 0.4 g	2.3	5.4	2.3	3.3

TABLE 11

Effects of Cellulase-Penicillin Combinations on Sludge Dewatering

100 ml samples of fresh sludge were treated as indicated below.

Sludge Treatment	θ/V^2	$\frac{\theta/V^2 \text{ Control}}{\theta/V^2 \text{ Treated}}$	Supernatants Replaced θ/V^2	 $\frac{\theta/V^2 \text{ Control}}{\theta/V^2 \text{ Treated}}$
Control	33.3	1.00	15.4	1.00
Cellulase 36 (1 g)	80.0	0.42	18.2	0.85
Cellulase 36 (1 g) + Penicillin (10,000 units/ml)	133.3	0.25	50.0	0.31
Cellulase 36 (0.5 g) + Penicillin (10,000 units/ml)	200.0	0.17	20.0	0.77

Table 12 presents the results obtained when various enzymes were added to streptomycin-treated sludge. With the exception of the streptomycin + pectinase sample, where it is now known that the high rate of filtration results from insoluble pectinase, there was a conspicuous increase in filtration rate associated with the amylase-containing sample, after supernatant replacement, and also with the streptomycin + lysozyme sample. The peculiar result found with streptomycin + hyaluronidase, namely a reduced filtration rate after supernatant replacement, may somehow be linked to the fact that this sample appeared thick and viscid when viewed in the phase microscope.

Flasks containing fresh sludge with lysozyme and either streptomycin or penicillin were prepared under aerobic conditions as indicated in Table 13. At 22 hrs, there were large numbers of free bacteria visible in microscopic preparations made from the penicillin + lysozyme and the streptomycin + lysozyme samples. Spirochaetes were absent from all the flasks except the control, where, strangely, they were inactive. The floccules appeared normal.

Table 14 shows the results of filtration, including those after supernatant replacement. The combination of lysozyme with either streptomycin or penicillin was disappointing as regards filtration. This is probably due to the aerobic environment in combination with the massive amount of enzyme used (see Discussion). Considerable improvement in the rate of filtration did occur after supernatant replacement. It is noteworthy that the filtration rate of lysozyme was unaffected by replacing its supernatant fluid.

Twenty-seven hours after the addition of the ingredients mentioned above, 0.5 g of papain was added to the flasks and incubation on the gyrotary shaker was continued for another 17 hr. At this time, many free cells could be seen in phase microscopic preparations of the antibiotic + enzyme samples. The penicillin + lysozyme + papain sample revealed a large number of spherical bodies which in all probability were protoplasts or spheroplasts. This is all the more likely in that a number of "crescents" were also seen; i.e., protoplasts/spheroplasts whose cytoplasm has been pushed to one part of the cell periphery by unequal osmotic forces. In addition, spherical "ghosts" were viewed; i.e., plasts that have lost their cytoplasm and now appear empty. To a lesser degree, this picture was repeated in the lysozyme + papain sample. There may be materials in the sludge, e.g., solubilized sludge matrix, that supply the necessary hypertonic environment required for the survival of plasts.

The experiments shown in Table 13 reveal that ordinary supernatant replacement appreciably affects the speed of filtration. Addition of papain markedly reduces the filtration rate, but if the samples are completely washed once, rather than merely having their supernatant fluids replaced, the filtration rate, especially in the case of streptomycin + lysozyme + papain, is increased; however, the lysozyme + papain sample was not improved by this procedure.

TABLE 12

Effects of Enzyme-Streptomycin Combinations - Shaker Cultures

Flasks containing 100 ml of fresh sludge and 1 g each of Streptomycin Sulfate, except the Control Flask, were treated with the following enzymes as indicated. The flasks were incubated at room temperature on a gyrotary shaker.

Sludge Treatment	θ/V^2	$\frac{\theta/V^2 \text{ Control}}{\theta/V^2 \text{ Treated}}$	Supernatants Replaced θ/V^2	Supernatants Replaced $\frac{\theta/V^2 \text{ Control}}{\theta/V^2 \text{ Treated}}$
Control	50	1.0	18.8	1.0
Streptomycin + α-Amylase (1 g) + β-amylase (0.1 g)	200	0.3	20.0	0.9
Streptomycin + Hyaluronidase (0.1 g)	200	0.3	150.0	0.1
Streptomycin + Lysozyme (1 g)	100	0.5	6.6	2.8
Streptomycin + Pectinase (1 g)	10	5.0	1.3	14.5

TABLE 13

Dewatering of Washed Treated Sludge - Shaker Culture

Flasks containing 100 ml of fresh sludge were treated with the antibiotics and enzymes indicated in the following amounts: Streptomycin Sulfate (1 g), Lysozyme (1 g), Papain (0.5 g). The Papain was added 27 hours after the other agents had been added. Flasks were incubated at room temperature on a gyrotary shaker. Supernatant replacement was performed 17 hours after the addition of the Papain.

Sludge Treatment	θ/V^2	$\frac{\theta/V^2 \text{ Control}}{\theta/V^2 \text{ Treated}}$	Supernatants Replaced	
			θ/V^2	$\frac{\theta/V^2 \text{ Control}}{\theta/V^2 \text{ Treated}}$
Control	40.0	1.0	16.7	1.0
Streptomycin + Lysozyme + (Papain)	100.0	0.4	9.5	1.8
Penicillin + Lysozyme + (Papain)			22.2	0.8
Lysozyme + (Papain)	100.0	0.4	12.5	1.3
Samples Washed 1 x				
Control	15.3	1.0		
Streptomycin + Lysozyme + (Papain)	6.1	2.5		
Penicillin + Lysozyme + (Papain)	16.6	0.9		
Lysozyme + (Papain)	12.4	1.2		

TABLE 14

Dewatering of Lysozyme-Antibiotic Treated Sludge - Shaker Culture

One g of 3 x crystallized Lysozyme was dissolved in each of the following flasks containing, respectively, Streptomycin Sulfate (1 g), Penicillin G Sodium (1 million units), in 100 ml of fresh sludge. Incubation was at room temperature on a gyrotary shaker.

			Supernatants Replaced	
Sludge Treatment	θ/V^2	$\frac{\theta/V^2 \text{ Control}}{\theta/V^2 \text{ Treated}}$	θ/V^2	$\frac{\theta/V^2 \text{ Control}}{\theta/V^2 \text{ Treated}}$
At 22 Hours				
Control	27.3	1.0	12.5	1.0
Streptomycin + Lysozyme	42.9	0.6	6.7	1.9
Penicillin + Lysozyme	100.0	0.3	11.1	1.1
Lysozyme	13.0	2.1	5.9	2.1

The potential value of lysozyme was borne out in an experiment in which lysozyme was omitted and papain (1 g) was added to both streptomycin and penicillin.

Microscopic examinations showed that yeasts started to appear in the treated samples by 22 hrs and that they increased rapidly in numbers. Filtration experiments were not carried out until the 5th day of incubation, at which time the plotted curves of the treated samples were almost perpendicular to the x-axis and slopes could not be obtained. Supernatant replacement had little effect on these filtration rates.

THE LYSOZYME-STREPTOMYCIN EFFECT

The marked improvement in dewatering rate observed when lysozyme was incubated with sludge under anaerobic conditions provided a basis for further investigations with this enzyme. The reproducibility of the phenomenon had to be established and improvements in performance, that could be brought about through antibiotic addition, had to be determined.

The interaction of the combined agents was tested in two ways, viz., in one series of experiments by keeping the streptomycin concentration fixed at 0.1 g and varying the amount of concomitantly added lysozyme, and in another test series by keeping the lysozyme level constant at 0.2 g and varying the amount of the added streptomycin. The details of the experiments are indicated in Table 15, where it may be seen that dewatering increased in the presence of a constant 0.1 g of streptomycin as the amount of lysozyme was increased; i.e., dewatering was more efficient in the flask containing 0.4 g of lysozyme than in that containing 0.1 g of lysozyme. Supernatant replacement appeared to reduce the filtration rate, but this was probably exaggerated because filtration was so fast. Evidently, here the increasing amounts of lysozyme were able to overcome or otherwise circumvent the apparent inhibitory action induced by the presence of greater amounts of streptomycin. It may be seen from Table 10 that an increased amount of streptomycin seemingly nullified the effect of a given constant amount of lysozyme; i.e., 0.2 g.

When the time required to collect 5 ml of filtrate was plotted against increasing amounts of streptomycin added to sludge (0.1, 0.2, 1 g), the curve seen in Figure 6 was obtained. Because of the improved efficiency of the dewatering process, the 5-ml collection times were so short in the present studies that they can, at best, only be regarded as indicating trends. With this reservation, Figure 6 shows that additions of streptomycin above 0.1 g/100 ml do not produce any appreciable gain in effect. Compared with Figure 7 for lysozyme alone, it is seen that further additions of streptomycin alone do not improve the dewatering process as much as further additions of lysozyme alone.

The lysozyme curve would seem to indicate that the lowest amount of this enzyme tested, viz., 0.1 g, produces the maximum effect and that further additions are superflous. Of course, if a concentration

TABLE 15

Improved Dewatering of Lysozyme-Streptomycin Combinations

Flasks containing 100 ml of fresh sludge were treated as indicated. The flasks were sealed with Parafilm and left undisturbed at room temperature until just prior to the filtration tests.

			Supernatants Replaced	
Sludge Treatment	θ/V^2	$\frac{\theta/V^2 \text{ Control}}{\theta/V^2 \text{ Treated}}$	θ/V^2	$\frac{\theta/V^2 \text{ Control}}{\theta/V^2 \text{ Treated}}$
At 20 Hours				
Control	14.3	1.0	4.8	1.0
Streptomycin 0.1 g + Lysozyme 0.1 g	5.6	2.6	4.0	1.2
Streptomycin 0.1 g + Lysozyme 0.2 g	4.0	3.6	3.3	1.5
Streptomycin 0.1 g + Lysozyme 0.4 g	3.7	3.9	2.1	2.3
At 21 Hours				
Control	10.0	1.0	8.3	1.0
Lysozyme 0.2 g + Streptomycin 0.2 g	4.8	2.1	3.6	2.3
Lysozyme 0.2 g + Streptomycin 0.5 g	6.7	1.5	4.8	1.7
Lysozyme 0.2 g + Streptomycin 1 g	9.1	1.1	4.8	1.7

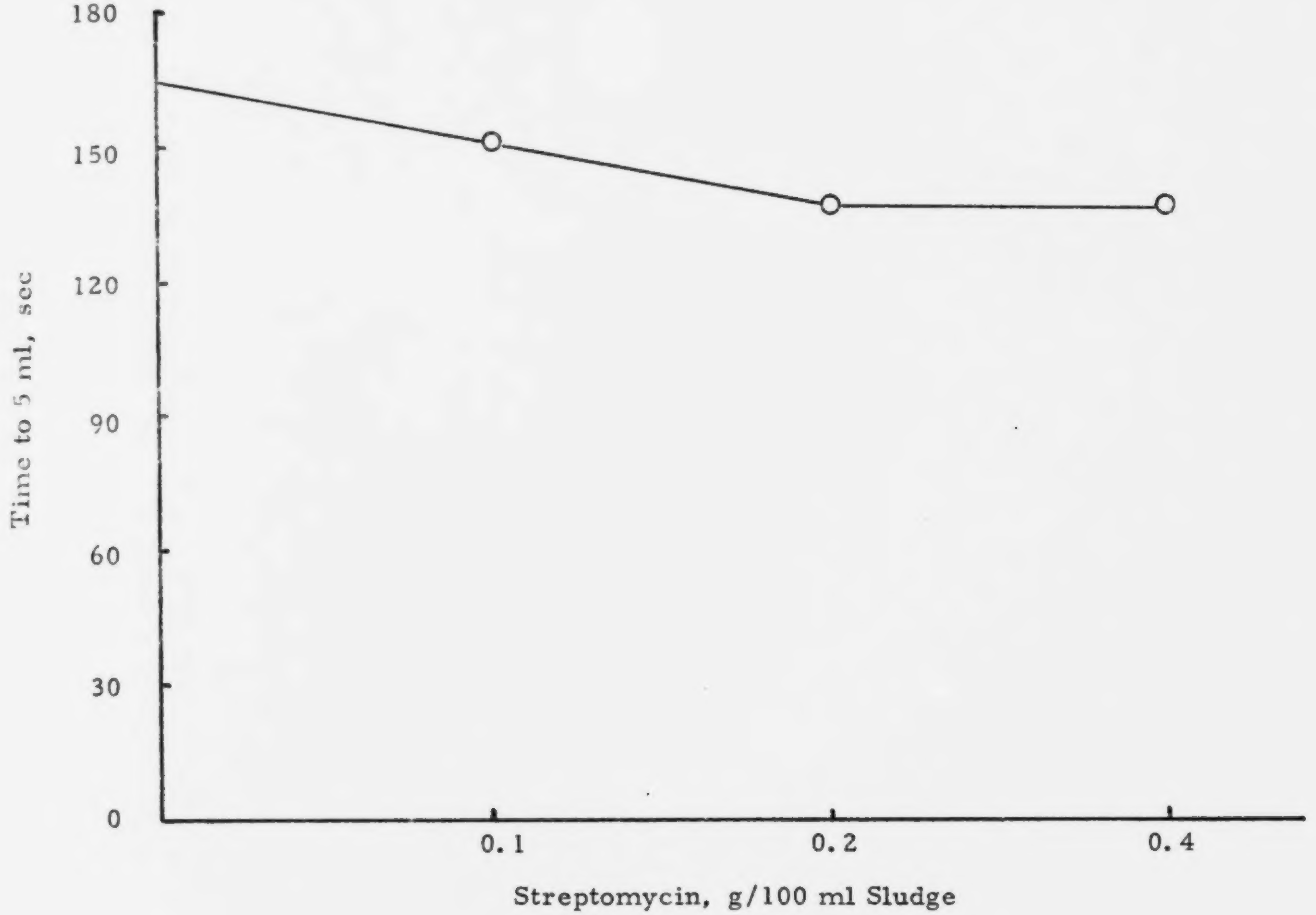

Figure 6. Effects of Streptomycin Concentration on Dewatering Rate after 23 Hour Incubation

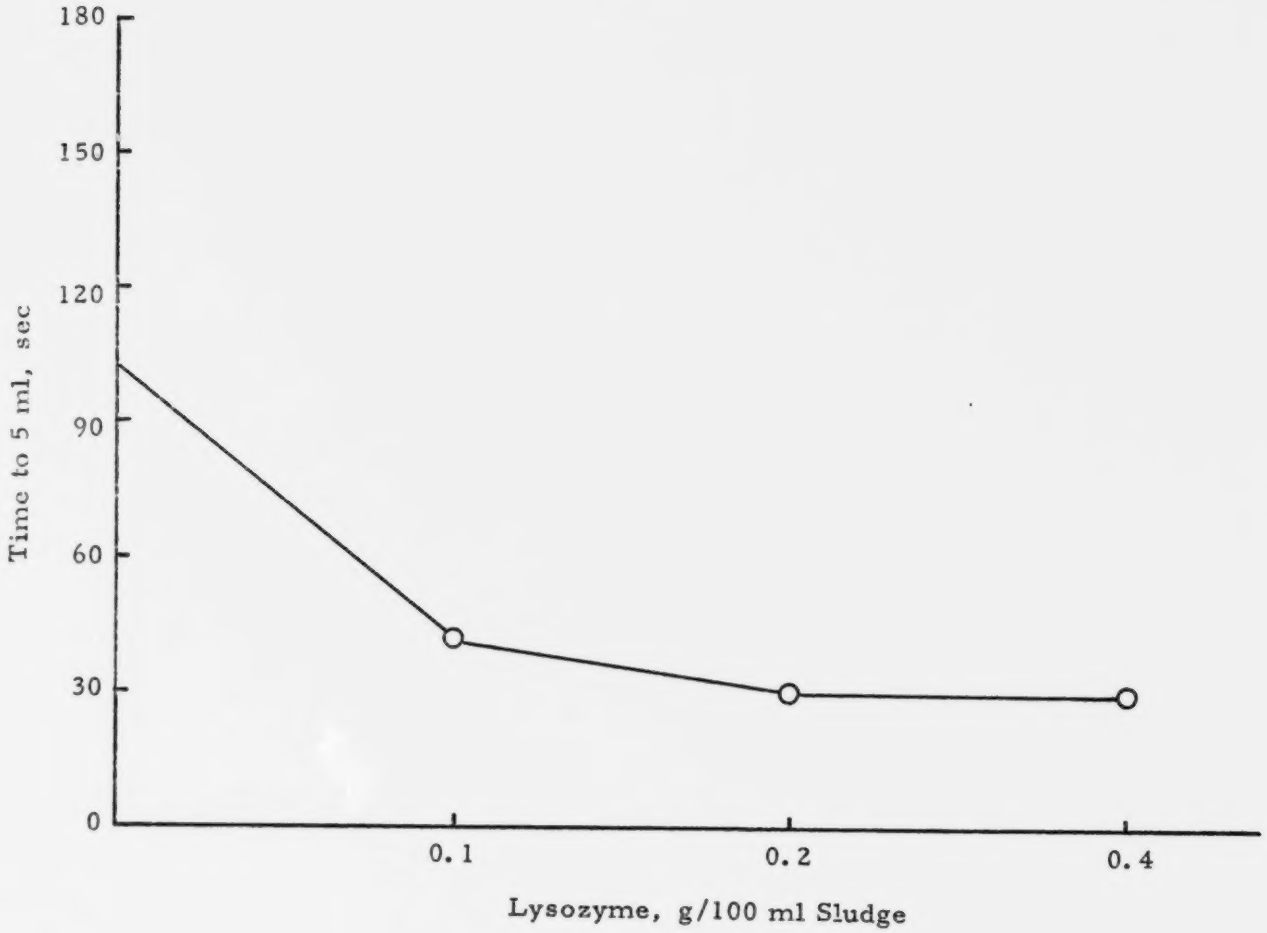

Figure 7. Effect of Lysozyme Concentration on Dewatering Rate after 22 Hour Incubation

of lysozyme below 0.1 g proves to be effective in dewatering, its relationship to streptomycin would have to be determined.

Figure 8 shows that in the presence of a constant amount of streptomycin (0.1 g), lysozyme at a concentration of 0.1 g produces the maximum effect and amounts of lysozyme beyond this are ineffective, probably indicating that the enzyme is saturated and functioning in a non-inhibitory way.

When lysozyme is kept constant (0.2 g) and the streptomycin varied (0.2, 0.5, 1 g), a plot of the 5-ml collection times gives the curve in Figure 9. Here the effect of the least amount of streptomycin is actually underplayed. Because the initial 30 sec volume obtained was 5.5 ml, and in that there was no way to extrapolate back, an arbitrary value of 5 was chosen for the 0.2 g streptomycin point, although this value presents the data in a poorer light than the true value would give. Overall, the curve indicates that in the presence of 0.2 g lysozyme, increasing the streptomycin beyond 0.2 g seems only to lead to increased inhibition by the streptomycin. Again, it is not known what the effect of increments of streptomycin would be if added to the optimal amount of lysozyme.

It is pertinent to mention here that microscopic examination of the above antibiotic and enzyme samples did not give any hint that the floc modification had occurred and such modification was only revealed by filtration tests.

A variation of the above experiment was carried out to verify if under anaerobic conditions the streptomycin is exhibiting an inhibitory action. An experiment to examine the role of the enzyme β-galactosidase was also included in this series. Table 16 shows the effects of these treatments on dewatering rates.

Attention is directed in Table 16 to the filtration rate resulting from the combined action of 0.1 g streptomycin and lysozyme. This combination resulted in a dramatic increase in the filtration rate of the sludge, being over 8 times that of the control material. The θ/V^2 value remained the same after supernatant replacement (even though the ratio appeared to change), indicating that this is a real effect and not due to an anomaly in the filtration set-up. The actual filtration was very rapid, the 10 ml sample running through in about 4 minutes with 9 ml of very clear supernatant recovered.

A greater amount of streptomycin, i.e., 1 g, is evidently inhibitory to the combined action of streptomycin and lysozyme in that the filtration rate in this case was less than a third that of the lower concentration (0.1 g) of streptomycin. The addition of β-galactosidase did not effectively increase the filtration rate in the presence of one gram of streptomycin.

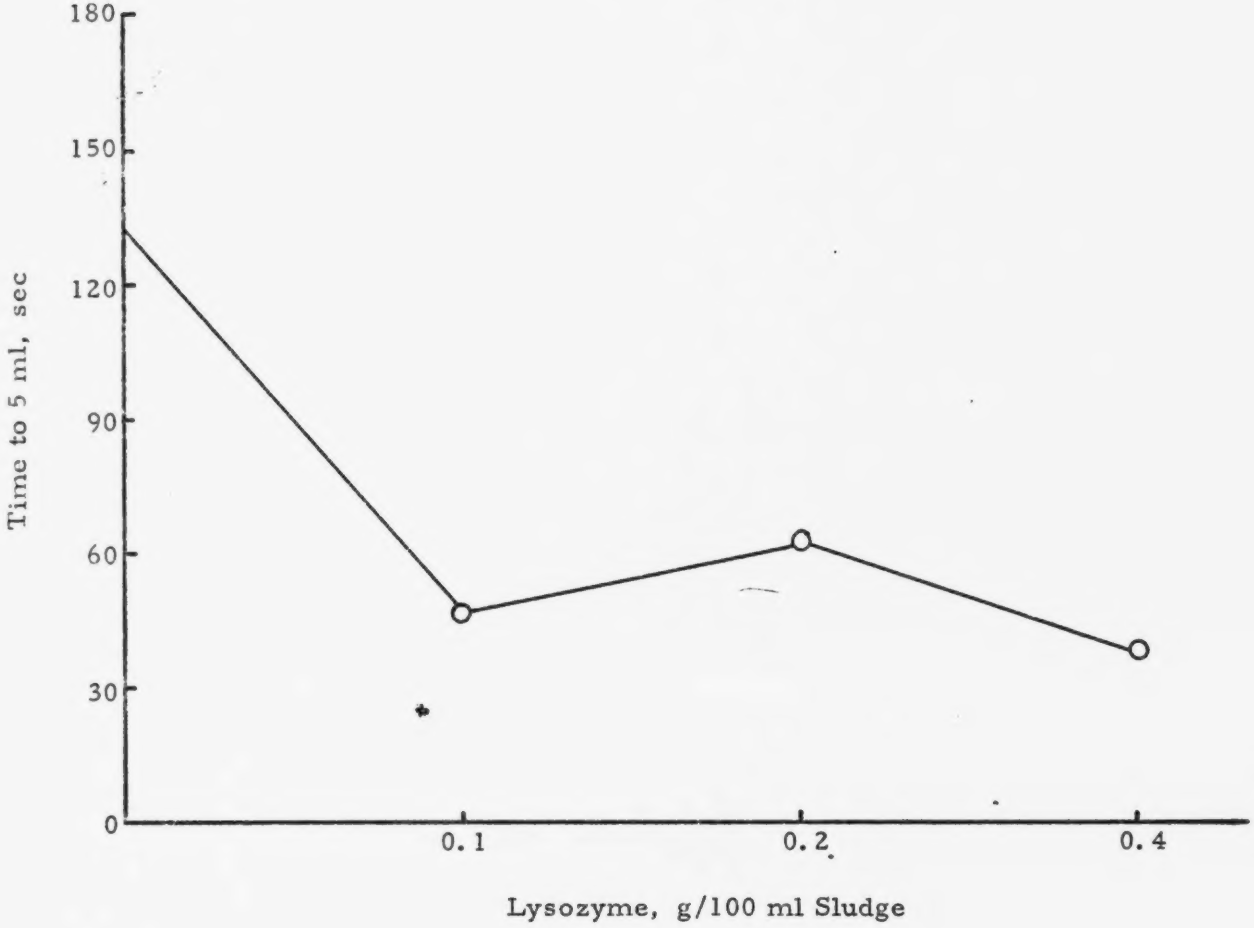

Figure 8. Effect of Lysozyme at Constant Streptomycin Concentration after 20 Hour Incubation

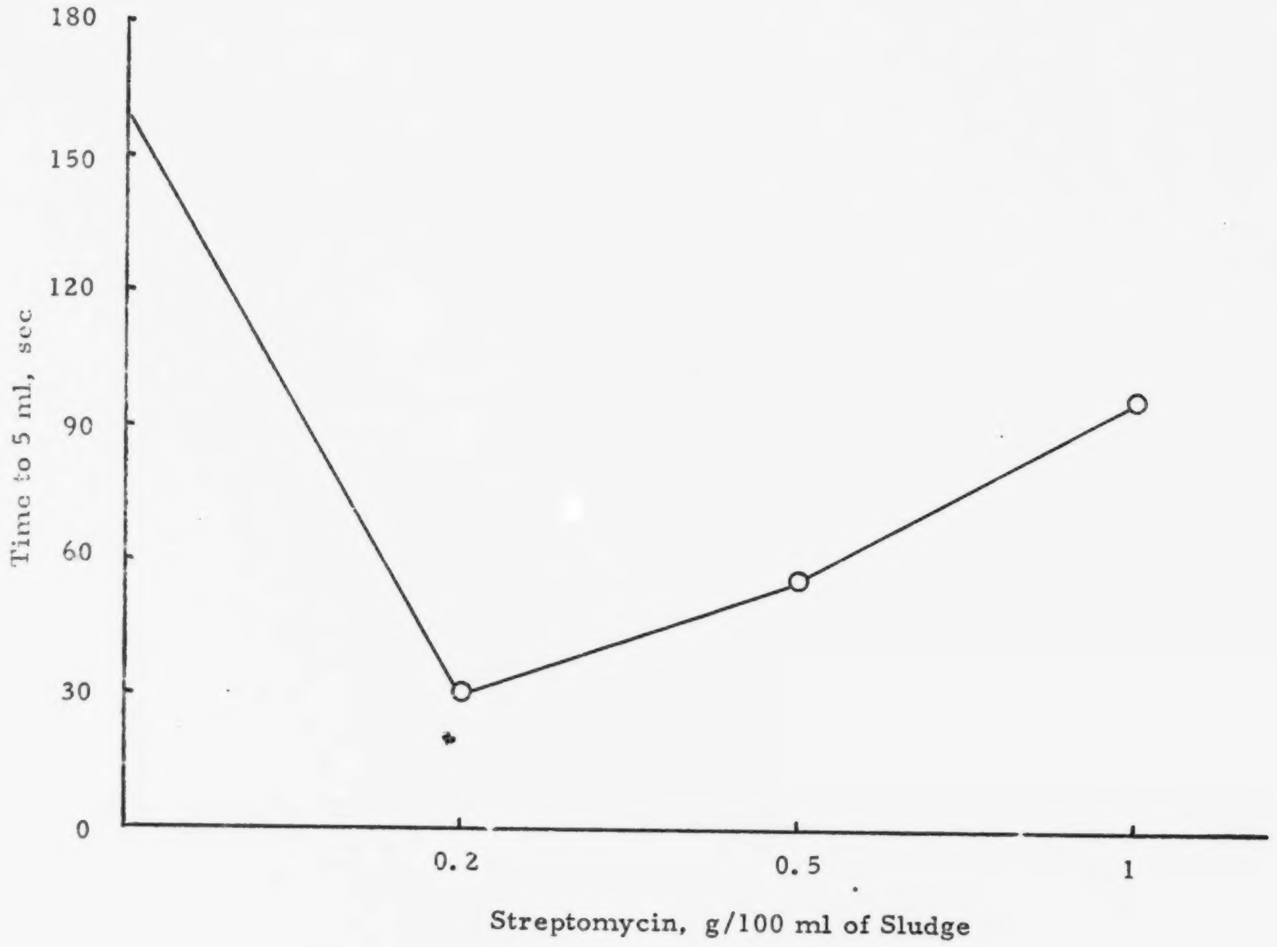

Figure 9. Effect of Streptomycin at Constant Lysozyme Concentration after 21 Hour Incubation

TABLE 16

Effect of Enzyme-Antibiotic Additions on Dewatering of Sludge

100 ml amounts of fresh sludge were set up as indicated. The Lysozyme (Sigma) concentration was constant at 0.2 g per flask. β-galactosidase (Worthington) was added as 1000 units; the concentration of Streptomycin was varied as indicated. The Parafilm covered flasks were incubated undisturbed as still cultures at room temperature.

			Supernatants Replaced	
Sludge Treatment	θ/V^2	$\frac{\theta/V^2 \text{ Control}}{\theta/V^2 \text{ Treated}}$	θ/V^2	$\frac{\theta/V^2 \text{ Control}}{\theta/V^2 \text{ Treated}}$
Control	16.6	1.0	16.7	1.0
Streptomycin (1 g) + Lysozyme	14.3	1.2	8.3	2.0
Streptomycin (0.1 g) + Lysozyme	1.9	8.7	3.4	4.9
Streptomycin (1 g) + Lysozyme + β-galactosidase	12.5	1.3	8.3	2.0

The rapidity of the filtration in the key experiments involving lysozyme was such that the quantitative improvement was actually being underestimated. A third series of tests was conducted in order to measure the additional parameters involved in characterizing a dewatering process. Accordingly, the experiment outlined in Table 17 was set up as indicated.

Here, the rate values obtained with lysozyme were significantly improved over the controls but the rapidity of the filtration of the relatively low solid sludge control once again prevented a higher ratio being measured.

A calculation of the per cent moisture remaining in the sludge cake is shown in Tables 18 and 19. The corresponding decrease in the sludge volumes presented in these tables compares the concentration effectiveness of the enzyme treatment.

Plots of the per cent moisture with time, as shown in Figures 10 and 11, reveal the rapid removal of moisture as a result of enzyme treatment. The dramatic change in filtration rate observed with lysozyme, as illustrated in Figure 11, indicates that there may be a strong correlation between initial sludge solids and enzyme activity.

The final filter cake solids at the end of 5 min of dewatering was 26.6% in the sludge with the 0.73% initial solids. In the sample with the 0.67% initial solids, the final filter cake at 5 min of dewatering contained 6.4% solids. The untreated sludge controls dewatered to only about 2% solids in the same time interval. The high cake solids (26%) compare favorably with those obtained by use of more conventional filter aids, such as $FeCl_3$, but is achieved without the objectionable increase in potentially toxic solids that have to be disposed of in subsequent processes. Time did not permit evaluation of the effects of enzyme treatment on the final cake solids when higher initial solids are used.

In order to provide information essential to pilot plant scale-up, the filtration resistance of the sludge was calculated, Table 20 (see Appendix A for method). This value can be used for comparison with other types of sludges whose resistance values are available.

On the basis of the filtration resistance, the theoretical yield of sludge solids that may be obtained from a conventional vacuum filter can be calculated (see Appendix A). The calculations presented here are based on actual experience with vacuum filtration obtained in a large activated sludge treatment plant. Pertinent assumptions included the following:

Proportion of time for which suction acts (remainder of time for cake removal) = 97%

Time for 1 drum revolution (drum diameter = 10 ft; length - 21 ft) = 2.5 min

Average pressure = 20 in. Hg

TABLE 17

Improvement in Sludge Dewatering by Lysozyme Treatment

Flasks containing 100 ml of fresh sludge were treated with Lysozyme and Streptomyin as indicated. The flasks were sealed with Parafilm and left undisturbed at room temperature until just prior to the filtration tests.

Note: Fifteen ml samples were used in the filtration tests.

Sludge Treatment	θ/V^2	$\frac{\theta/V^2 \text{ Control}}{\theta/V^2 \text{ Treated}}$
Control	9.1	1.0
Lysozyme (0.2 g) + Streptomycin (0.1 g)	2.6	3.5
Lysozyme (0.2 g)	2.1	4.3
Lysozyme (0.1 g) + Streptomycin (0.1 g)	4.5	2.0

TABLE 18

Reduction of Sludge Volume and Changes in Solids Concentration During Vacuum Filtration

Time, Min	Sludge Solids %		Sludge Moisture %		Amount of Original Volume Remaining %	
	Lysozyme	Control	Lysozyme	Control	Lysozyme	Control
0	0.73	0.73	99.27	99.27	100	100
1	1.82	1.45	98.18	98.55	40	50
2	2.76	1.60	97.24	98.40	26	45
4	8.00	1.86	92.00	98.14	9	39
5	26.60	1.96	73.33	98.04	3	37

TABLE 19

Reduction of Sludge Volume and Changes in Solids Concentration During Vacuum Filtration

Time, Min	Sludge Solids %		Sludge Moisture %		Amount of Original Volume Remaining %	
	Lysozyme	Control	Lysozyme	Control	Lysozyme	Control
0	0.67	0.67	99.33	99.33	100	100
1	1.41	1.00	98.59	99.00	47	67
2	1.96	1.10	98.04	98.90	34	60
5	6.47	1.40	93.53	98.10	10	47
Filter Cake	0.71	0.54				
Filtrate Solids	0.06	0.08				
Filtrate Turbidity O.D.	0.036	0.033				

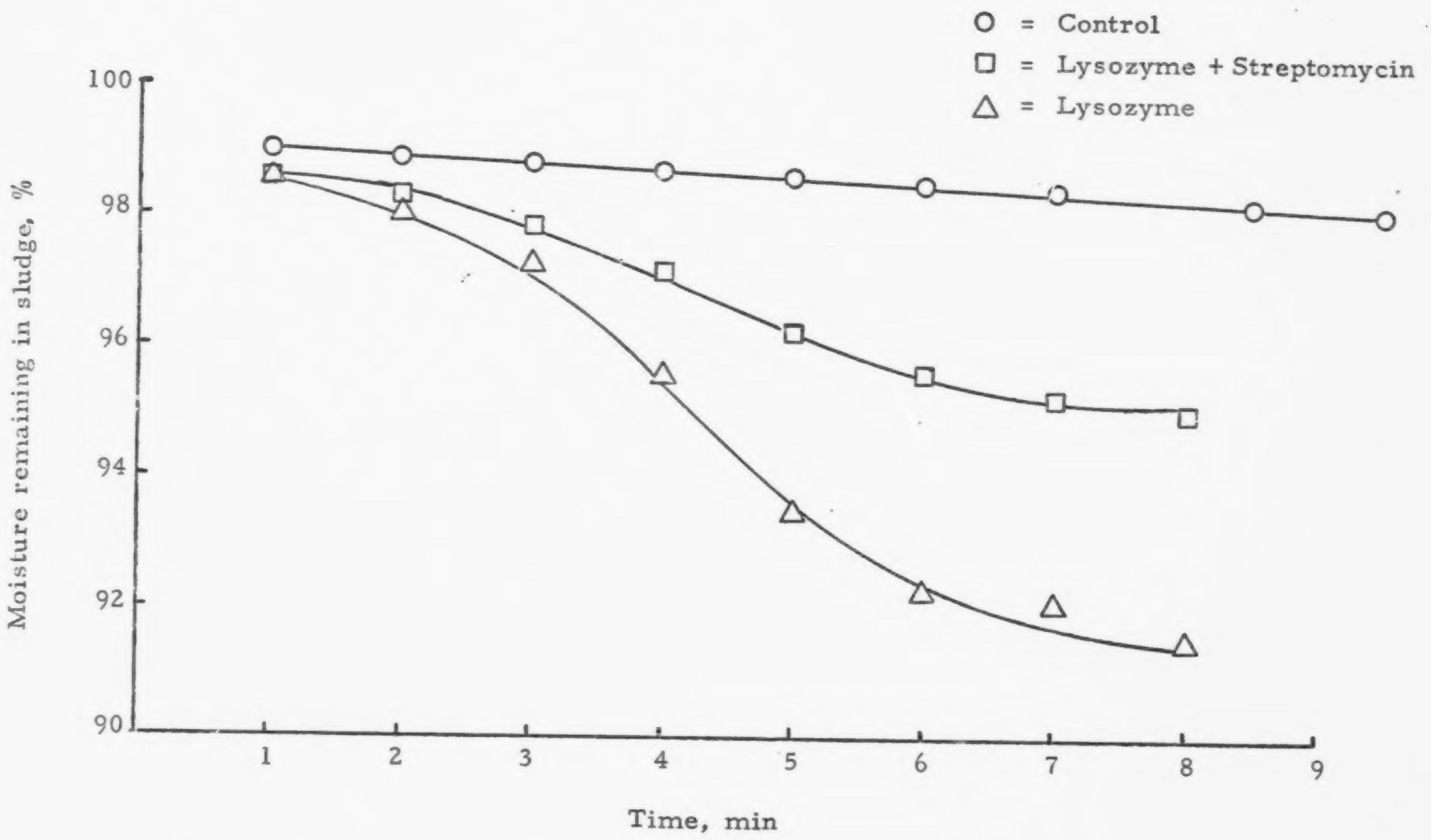

Figure 10. Rates of Release of Moisture by Lysozyme Treated Sludge
0.67% Initial Sludge Solids
Lysozyme concentration = 0.2 g/100 ml sludge
Streptomycin concentration = 0.1 g/100 ml sludge

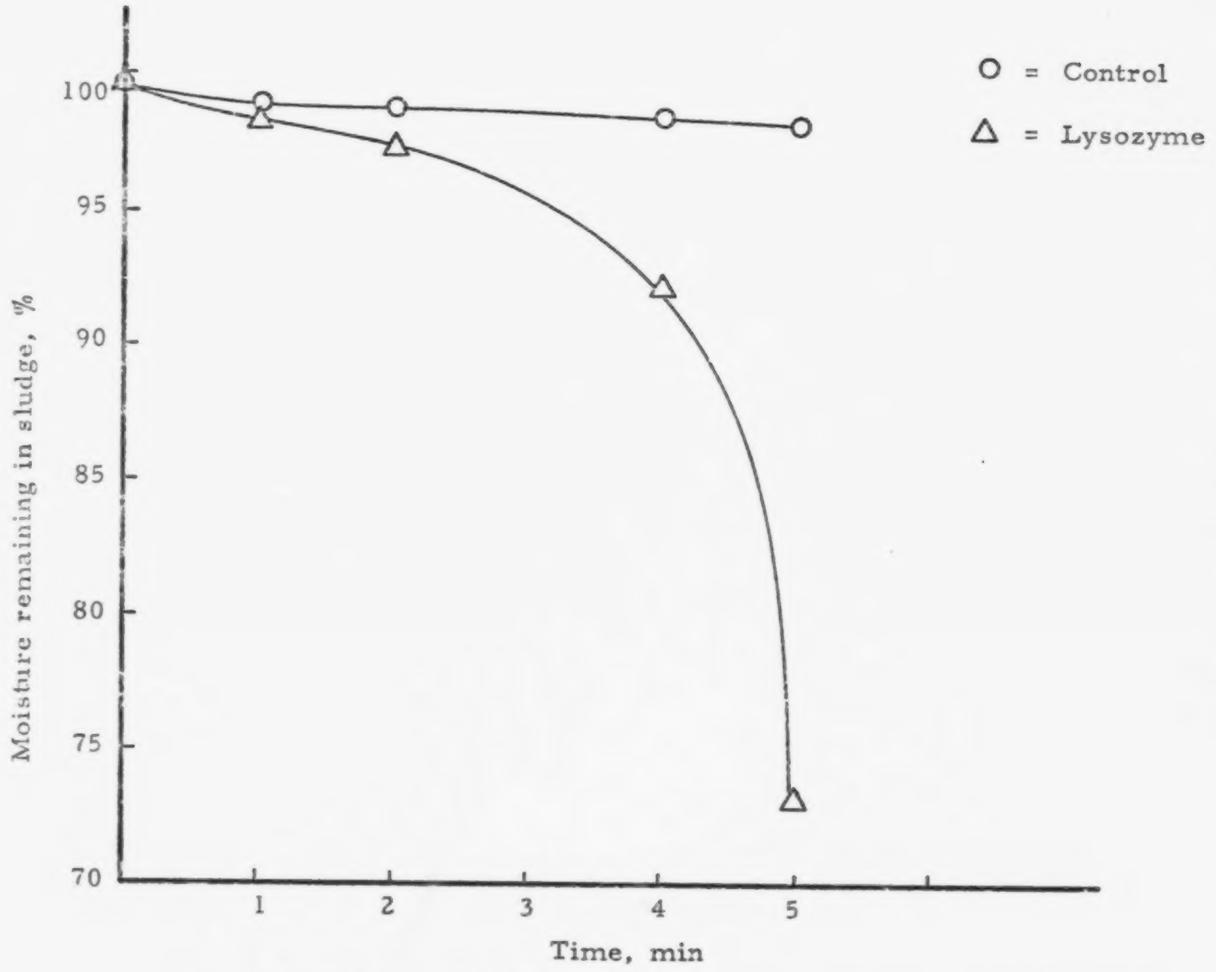

Figure 11. Rate of Release of Moisture by Lysozyme Treated Sludge
0.73% Initial Sludge Solids
Lysozyme concentration = 0.2 g/100 ml sludge

Table 20 presents the expected yield of dried cake solids from the experiments involving lysozyme presented in Tables 18 and 19. Two examples are used: one example illustrates the expected yield when an 80% final moisture content is desired, the other when a 92% final moisture content is sought. The improved solids yield per hour from a vacuum filter, as a result of treatment, is more than double that of the untreated sludge in both cases. The lower filter cake yield obtained by reducing the moisture content from 92% - 80% is slight, reflecting the fact that only a small amount of additional water had to be removed to achieve large increases in solids, as displayed in Figure 11.

INFLUENCE OF POLY-β-HYDROXYBUTRIC ACID DEPOLYMERASE OF SLUDGE FILTERABILITY

PHBA Addition to Sludge

To test for any possible effect of the PHBA depolymerase on the filtration rate of sludge, a relatively large amount of this enzyme was incubated with slightly concentrated sludge, as shown in Table 8. It can be calculated from the data in the methods section that the amount of enzyme used would saturate 6.5 mg of poly-β-hydroxybutyric acid. This amount of polymer represents 5% of the solid organic matter in the reaction mixtures, and is equivalent to that previously determined to be in the sludge (Table 1). If all of this polymer were accessible to the enzyme, it would be completely degraded in 10 min at 23°C. To allow for a possibly reduced rate of depolymerase activity in the presence of sludge, the reaction mixtures were incubated for 30 min. They were then diluted in buffers at pH values ranging from 3.6 to 9.0, and the filtration rates were tested. There appears to have been no real change in the filtration rate at any of the pH values tested, although there is an indication that the enzyme-treated samples may have filtered somewhat more slowly than the untreated samples.

Effects of Sludge on the Activity of Poly-β-Hydroxybutyric Acid Depolymerase

It is possible that inhibitors in the sludge inactivated the PHBA depolymerase, or rendered any polymer unavailable for enzymatic digestion. If such inhibitors were present, the rate at which exogenous polymer would be digested by exogenous depolymerase in the presence of sludge might be reduced. To examine this question, an experiment designed to reveal any striking inhibitory effects was carried out. As shown in Table 21, the supernatant liquid contains an unidentified material that interferes with the colorimetric assay for β-hydroxybutyric acid. This material appears to be associated with the enzyme fraction and seems to increase with time when either sludge or its supernatant liquor is incubated with the depolymerase. The material is not the polymer in that the assay used reveals total β-hydroxybutyryl moieties in solution and therefore no variation should be found with increasing incubation. The average quantity of this material corresponds to 50 μg "hydroxybutyrate"/ml.

TABLE 20

Predicted Solids Yield from a Vacuum Filter

Values obtained from two separate experiments involving sludge treated with lysozyme and streptomycin and lysozyme alone.

	Control	Streptomycin (0.1 g) + Lysozyme (0.2 g)	Relative Increase in Yield of Solids	Control	Lysozyme (0.2 g)	Relative Increase in Yield of Solids
% Solids	0.73	0.73		0.67		
Specific filtration resistance (r)	6.9×10^9	8×10^8		2.17×10^9	5×10^8	
Yield of dry cake solids (L) at: 80% moisture	0.26	0.75	2.91	0.42	0.89	2.1
92% moisture	0.27	0.80		0.46	0.96	

L = lbs of solids per sq ft of filter area per hr.

r = $\sec^2$ per gram.

TABLE 21

Effect of Sludge on Digestion of Poly-β-Hydroxybutyrate by Depolymerase

Final reaction mixtures contained 5.0 ml of sludge concentrated to 5.1% (w/v) solids by centrifugation, 7.5 ml 0.05 M pH 8.0 Tris-HCl, 1.0 ml crude depolymerase, 0.2 ml polymer containing 162μ equivalents β-hydroxybutyric acid/ml of reaction mixture, and H_2O to a final volume of 15.0 ml. At intervals after starting the reaction, 1.0 ml samples were removed and added to 3.0 ml H_2O at 2°C to stop further digestion. The chilled samples were centrifuged and all soluble reaction products in the supernatant liquid were measured as β-hydroxybutyric acid by the method of Slepecky and Law (1960).

Sludge Treatment (30 min, 23°C)	Reaction then Initiated by Adding to the		Soluble reaction products as μg β-hydroxybutyric acid/ml Time Samples, min		
	whole sludge	sludge liquid	10	30	60
No addition	enzyme	-	44	64	60
No addition	-	enzyme	32	40	64
No addition	-	enzyme + polymer	200	224	214
No addition	enzyme + polymer	-	136	174	174
Enzyme	-	polymer	104	136	174
Enzyme	polymer	-	152	220	188
Polymer	enzyme	-	128	180	184

In the presence of the supernatant liquid of sludge, the enzymatic digestion of polymer proceeded rapidly and to completion. Thus, 162 g of β-hydroxybutyric acid as polymer/ml was added, and 164 μg/ml (or 214 μg/ml less the background material of 50 μg/ml) were recovered at the end of the experiment. In ~~contrast, when the recovered at the end of the experiment~~. In contrast, when the reaction was allowed to take place in the presence of whole sludge, the rate of depolymerization was reduced, and not all the added polymer was digested by the end of the experiment.

The reason for this reduction in rate is not difficult to explain. It may be noted that when sludge was incubated with either polymer or enzyme for 30 min before adding enzyme or polymer respectively to start the reactions, the rates of digestion were equivalent to that observed when enzyme and polymer were added simultaneously. However, when the sludge was incubated with enzyme and centrifuged, the rate of polymer digestion by enzyme remaining in the supernatant liquid was even slower.

These observations are not inconsistent with previous work (Delafield (1964); Doudoroff (1966)). The depolymerase is a basic protein and a portion of it may become rapidly adsorbed to anionic surfaces in the sludge. This portion would then be unavailable to digest the polymer and the rate of depolymerization would accordingly decrease. The kinetics of this process were not studied in more detail. However, as an approximation, it would appear that inactivation of the depolymerase is slow and incomplete. Accordingly, the negative effect of depolymerase on filtration rate cannot be attributed to inactivation of the enzyme. The possibility that poly-β-hydroxybutyric acid exists in the sludge in a form which the depolymerase digests slowly (as for example occurs when polymer precipitated from chloroform by ethanol is used as a substrate) was examined by performing the same experiment shown in Table 22, but with an incubation period of 16 hours. The same negative results were again obtained.

Effects of PHBA-Depolymerase on the Filtration Rate of Sludge Previously Treated with Trypsin and Peptidase

The inability of the depolymerase to solubilize compounds of β-hydroxybutyrate from the sludge could, in principle, be attributed to inactivation of any poly-β-hydroxybutyrate in the sludge. Poly-β-hydroxybutyric acid is conventionally termed inactive when the surface of the polymer granules is occluded by adsorption of non-specific proteins. The observation that the sludge appears to contain some hydrolyzable protein suggested that polymer in the sludge might well be coated. To test this possibility, the experiment shown in Table 23 was performed. The sludge was incubated with trypsin at a final concentration of 1.0 mg/ml for three hours. At the end of this period the residual tryptic activity was negligible, presumably owing to self-digestion of the enzyme. Depolymerase was then added to the trypsinized sludge, and incubation

TABLE 22

Effect of Depolymerase on the Filtration Rate of Sludge

Fresh sludge was concentrated to 1.4% (w/v) solids by settling. Reaction mixtures contained 10 ml sludge, 3.0 ml crude depolymerase, and 2.0 ml $MgCl_2$ to a final concentration of 10^{-3} M. Control mixtures were made with 3.0 ml H_2O in place of the enzyme. After 30 min at 23°C, the mixtures were diluted with (1) 15.1 ml 0.1 M pH 9 Tris-HCl; (2) 15.1 ml 0.1 M pH 7 phosphate; (3) 15.1 ml 0.1 M acetate; or (4) 15.1 ml 0.1 M pH 3.6 acetate. The filtration rates were then determined.

A Additions to reaction mixture	B pH		C θ/V^2	D(a) $\frac{\theta/V^2 \text{ in } H_2O}{\theta/V^2 \text{ in enzyme}}$
	During incubation	During filtration		
H_2O	7.4	9.0	26	-
"	7.4	7.0	42	-
"	7.4	5.0	35	-
"	7.4	3.6	30	-
Depolymerase	7.4	9.0	46	0.58
"	7.4	7.0	59	0.72
"	7.4	5.0	62	0.56

(a) Determined from the rates in column C at comparable pH values of filtration.

TABLE 23

Vacuum Dewatering Rates of Sludge Treated with Trypsin and Peptidase and then Treated with PHBA Depolymerase

Fresh sludge was concentrated to 2.89% solids. Reaction mixtures contained 6.5 ml sludge, 3.5 ml 0.1 M Tris pH 8.0 and 1.5 mg/ml Trypsin and 0.5 mg/ml peptidase. Suspensions incubated for one and two hours. At one and two hrs, pH's were adjusted for vacuum filtration with the following buffers: (1) 10.0 ml 0.1 M Tris pH 9; (2) 10.0 ml 0.1 M PO_4 pH 7.0; (3) 10.0 ml 0.1 M acetate pH 5.0; and (4) 10.0 ml 0.1 M acetate pH 3.6. The filtration rates were then determined. θ/V^2 = slope of the filtration rate.

Additions to Reaction mixture	pH		θ/V^2		θ/V^2 in H_2	
					θ/V^2 in enzyme	
	During Incubation	During Filtration	1 hour	2 hours	1 hour	2 hours
H_2O	8.0	9.0	145	212	-	-
"	8.0	7.0	425	275	-	-
"	8.0	5.0	320	352	-	-
"	8.0	3.6	164	114	-	-
Depolymerase	8.0	9.0	340	350	0.43	0.61
"	8.0	7.0	350	380	1.12	0.72
"	8.0	5.0	352	380	0.91	0.93
"	8.0	3.6	230	187	0.72	0.61

was continued. The mixture was samples at one and at two hours after adding the depolymerase, and the filtration rates were compared with those of sludge samples from which depolymerase had been omitted. As can be seen in Table 23, the depolymerase had no detectable effect on the filtration rate.

The inability to improve sludge dewatering utilizing the PHBA depolymerase intimated that the form of the polymer may be different and/or more complex than had been assumed. Since other types of bacterial polymers are known, it was decided to screen available enzymes whose activity is specific for these polymers. Accordingly, a screening procedure was developed incorporating various conditions of pH of filtration, pH of incubation, and time of incubation.

No significant improvements in filterability were observed as a result of this screening. Detailed descriptions of these experiments and the filtration rate data are presented in Appendix B.

SECTION VI

THE SLUDGE ENVIRONMENT

Many enzymes exist that are able to degrade organic polymers as complex as those suspected to exist in sludge. These enzymes may be elaborated under conditions that differ greatly from those found in the sludge environment. Thus a major effort to screen enzymes that are known to attack complex polymers and to isolate matrix-specific enzymes and include them in the screening program became of signal importance.

Enzymes are changed and/or inactivated by the same agents and conditions that denature other proteinaceous substances. To assure the survival and activity of enzymes in a metabolically competitive situation it is imperative that the sludge environment be defined.

Hence, it was important to measure the amount of proteinaceous material that may be present in the sludge, since lysis of microbial cells would release enzymes, proteins, amino acids and other compounds that might interfere with the activity of exogenous enzymes. In addition, the amount of cellular breakdown could indicate what levels of internal polymeric materials, of possible significance in flocculation, may be released from the cells.

AMINO ACID CONTENT OF SLUDGE SUPERNATANTS

A measure of the degree of cell lysis that occurs in sludge is the amount of protein found in the supernatant liquor. Since proteolytic enzymes are also released, it could be expected that some of these proteins would be converted to amino acids. To test this possibility, the supernatant liquid of fresh sludge, from which solids were removed either by centrifugation or settling, was analyzed for tyrosine. As shown in Table 24, the solutions contained material corresponding to roughly 3μg "tyrosine"/ml. This material, furthermore, must originate from the sludge solids, for when the sludge was concentrated to roughly 5% dry weight and incubated at 23° for two or three hours, the amount of "tyrosine" in the supernatant liquid increased to 30-40 μg/ml.

In the following discussion, soluble amino acids are reported, with reference to the standard, as tyrosine. It should be emphasized that this method measures only a portion of the total amino acids. As a generalization, the total amino acid concentration by weight in a protein hydrolysate is roughly twenty times that of the tyrosine and tryptophan concentrations.

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TABLE 24

Amino Acids in the Supernatant Liquid of Fresh Sludge

Fresh sludge containing 0.5% solids was centrifuged at 12000 x g for 10 min or settled at 1 x g for 120 min. The supernatant liquid from the settled sludge contained traces of solid material, and was further clarified by centrifugation. Both solutions were stored at 5°C for 4 hours. A third sample of the sludge was centrifuged and the sediment was resuspended to 4.0% solids in a small volume of the supernatant liquid. After incubation for 190 min at 23°C the concentrated sludge was again centrifuged, and the supernatant solution was saved. All three supernatant solutions were then analyzed for tyrosine using a calorimetric procedure for total amino acids using ultraviolet absorption, before and after addition of TCA, as described in the test. All values are reported with reference to the undiluted solutions.

	B	C	D	E	F	
					Total Amino Acids	
	Dilution in	O.D.	O.D	Tyrosine	Colorimetric 1	UV absorption 2
Source of Solution	5.0% TCA	260 nm	280 nm	mg/ml	mg/ml	mg/ml
Centrifuged, dilute sludge	0	0.141	0.120	2.7	0.054	0.079
Settled, dilute sludge	0	0.142	0.118	2.3	0.046	0.075
Centrifuged, concentrated sludge	0	1.725	1.344	34.0	0.680	0.720
Centrifuged, dilute sludge	1:3	0.196	0.132	1.5	0.030	0.073
Settled, dilute sludge	1:3	0.171	0.114	0.8	0.016	0.048
Centrifuged, concentrated sludge	1:3	-	-	33.0	0.660	-

F1: calculated from column E by assuming that tyrosine and tryptophan represent 5% by weight of total amino acids.

F2: calculated from the formula: - mg protein or amino acids/ml = 1.55 x OD minus 0.76 x $OD_{260\ nm}$. About 7% by weight of this material is nucleic acid or nucleotides (Warburg and Christian, loc. cit.).

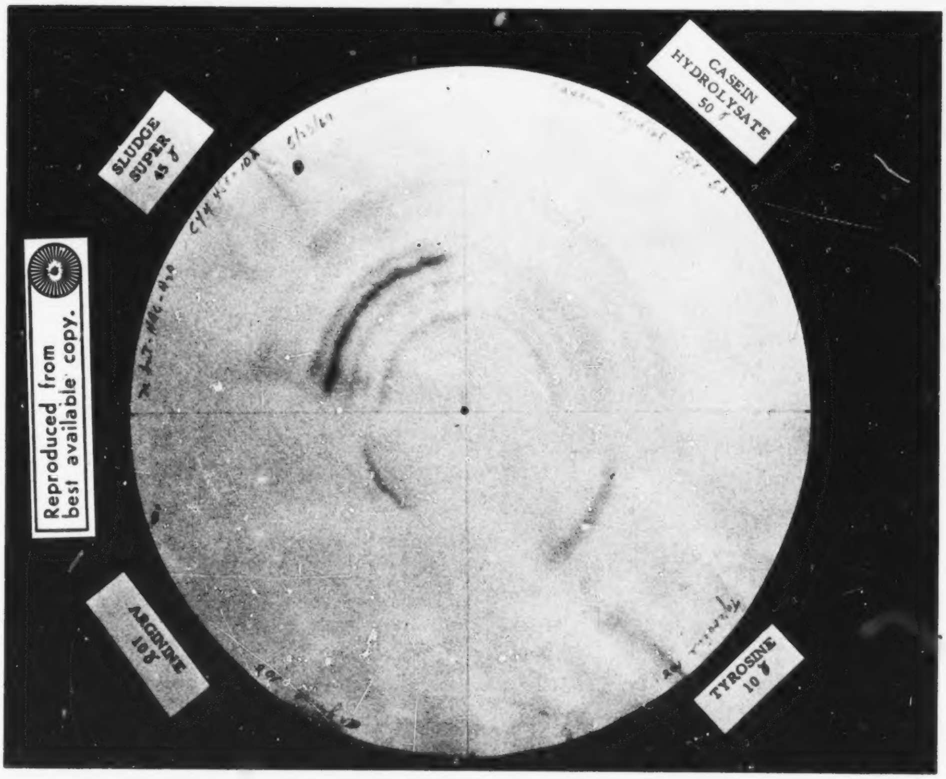
OD = optical density.

A clue that these reactions can actually be attributed to tyrosine (and tryptophan), rather than to some unknown compound, was obtained by examining the absorption of the solutions at 260 nm and 280 nm. According to the method of Warburg and Christian (1941), the amount of protein and nucleic acid in the solutions may be calculated from the absorbencies. As shown in Table 24, the values of protein obtained spectrophotometrically are in fair agreement with those obtained colorimetrically, assuming a tyrosine and tryptophan content of 5% by weight. Furthermore, a blue color develops when the solutions are spotted on filter paper and sprayed with ninhydrin, as would be expected if amino acids were present. Finally, Table 24 illustrates the fact that after the solutions are mixed with trichloracetic acid (TCA) and filtered, there is little or no decrease in the amount of reactive material in the filtrate. This observation indicates that the supernatant liquid of sludge contains free amino acids and nucleotides, rather than protein and nucleic acid as would be expected if mass lysis were occurring. In the actual experiment, the tyrosine content of the dilute sludge samples is lower in the presence than in the absence of TCA; the reason for this is that addition of the TCA reduced the concentration of tyrosine in those samples to a level that was difficult to estimate with any degree of accuracy.

As pointed out in the introduction, proteins may coat the surface of the poly-β-hydroxybutyrate particles shielding them from the added PHBA depolymerase. Verification of the presence of the amino acids and the absence of proteins was thus important. Knowing the qualitative makeup of the amino acid mixtures gave some clues as to their source and the metabolic processes that were occurring. To examine these points in more detail, sludge was concentrated to 4% solids and incubated at 23°C. At intervals, samples were taken from the mixture and centrifuged. The supernatant solutions were analyzed by radial chromatography on Whatman #1 Filter paper according to the method of Giri (1953). The solutions were applied near the center of the paper, and the solvent (butanol: acetic acid: water - 10:2. 2:5 by volume) was drawn from a beaker through a wick inserted at the center of the paper. Papers of 12 cm diameter were developed for one hour, dried at 80°C, sprayed with ninhydrin, and warmed at 80°C to promote the reaction between ninhydrin and amino acids.

The chromatogram shown in Figure 12 indicates that a spectrum of amino acids, resembling that seen in commerical lactalbumin hydrolysate, is present in the supernatant liquid of sludge. Furthermore, when the hydrolysate is diluted to a concentration of amino acids equivalent to that estimated to be in the sludge liquid, the bands formed from equal quantities of both solutions have approximately equal intensity.

The incomplete resolution of the chromatogram does not permit the conclusion that sludge liquid contains all of the naturally occurring amino acids. Some, indeed, may be absent from it to judge from the fact that the band nearest the origin in Figure 12 is considerably fainter


SLUDGE SUPER 45 γ
CASEIN HYDROLYSATE 50 γ
ARGININE 10 γ
TYROSINE 10 γ

Reproduced from best available copy.

Figure 12. Circular chromatogram of sludge supernatant. Amino acid pattern in sludge supernatant liquor is compared to a casein hydrolysate and to the individual amino acids arginine and tyrosine. There is a strong suggestion that arginine is absent from the sludge liquor.

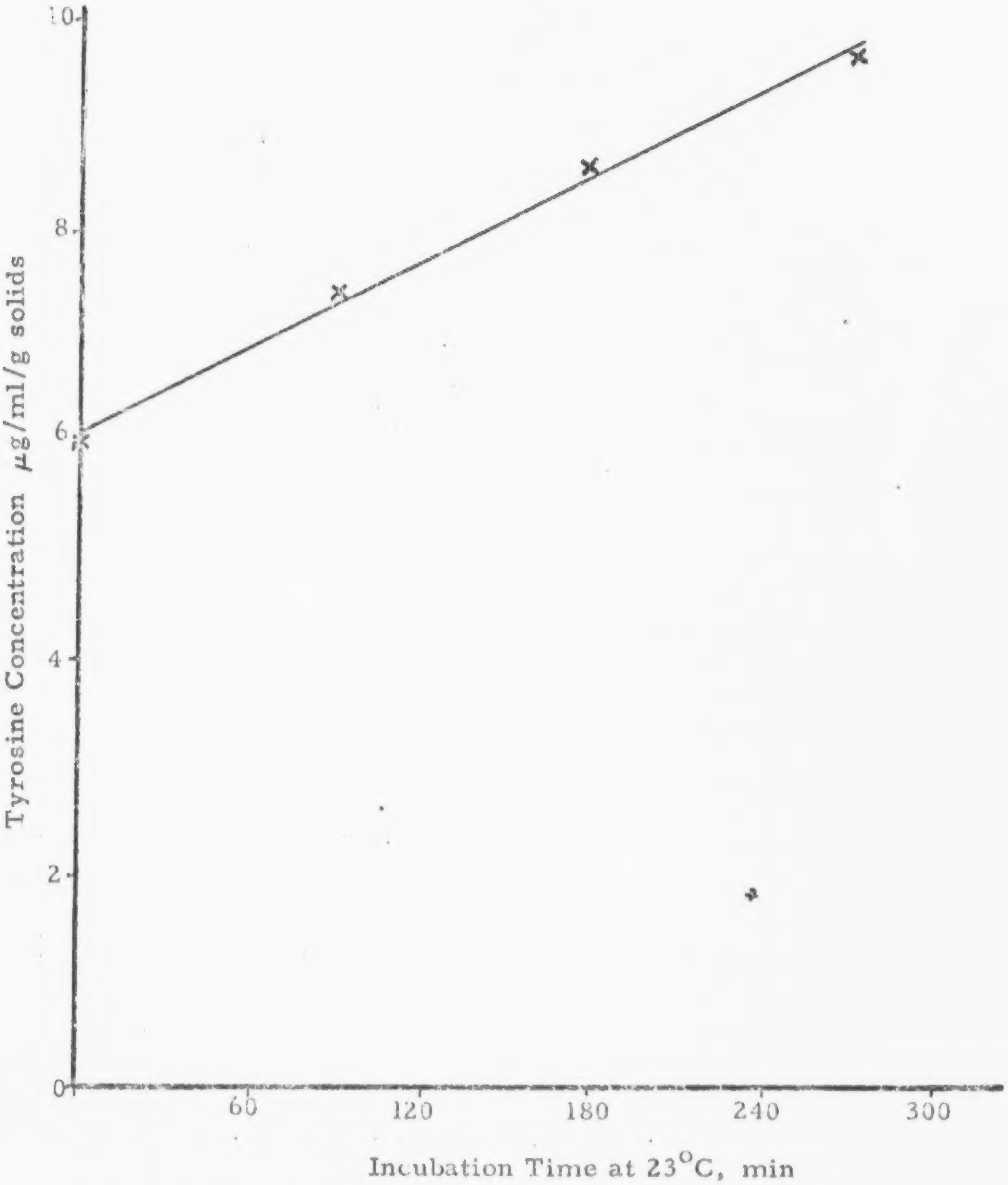


Figure 13. Solubilization of Tyrosine from Sludge Concentrated to 4% (w/v) Solids

TABLE 25

Comparison of Cell Free Organic Matter in Incubated Sludge Supernatant by Gravimetric and Colorimetric Determinations

Fresh sludge was concentrated to 3% solids and was incubated at 23° for 180 minutes. The sludge was then centrifuged, and the supernatant liquid was weighed after heating progressively at 105°, 190°, and 600°C. Amino acids and nucleotides in the supernatant liquid were estimated by the method of Warburg and Christian (1941).

	Gravimetric g/ml	Colorimetric g/ml	% of total organic weight
Solids at 105°C	1200	-	-
Water of hydration removed at 190°C	144	-	-
Ash remaining at 600°C	746	-	-
Amino acids	-	256	82%
Nucleotides	-	28	9%
Total organic material recovered	310*	284	91%

*Determined by subtracting ashed weight from weight after heating at 190°C.

in Super I and Super II than in the Hydrolysate. This band would contain arginine, an amino acid that may be absent in sludge supernatant since when the chromatogram was sprayed with the Sakaguchi reagent a brown color appeared here, rather than the typical organge color of arginine (MacPherson (1942)). However this may be, it is apparent that sludge liquid contains free amino acids or oligopeptides, but little or no proteins, which would have remained as a ninhydrin-stainable spot at the origin had they been present.

LEAKAGE OF AMINO ACID POOLS

As has been mentioned, it is evident that these amino acids must arise from the sludge solids, for their concentration in solution is proportional to the concentration of solids in suspension, as shown in Table 24. To examine the kinetics of their formation, concentrated sludge was incubated at 23°C; at intervals, samples of the mixture were centrifuged, and the tyrosine in the supernatant liquid was measured. As shown in Figure 13, the solubilization of tyrosine appears to occur at a steady, slow rate unaffected by the manipulations involved in concentrating the sludge.

At least two explanations for the slow solubilization of tyrosine shown in Figure 13 can be advanced: (1) per unit time, a small fraction of the cellular population of the sludge lyses, and the protein released is rapidly hydrolyzed; (2) there is a constant slow leakage of amino acids through the membrane of some or all of the cellular population.

To determine whether soluble organic compounds other than amino acids and nucleotides were present, fresh Pomona sludge was concentrated by centrifugation and then resuspended in a small amount of the supernatant liquid. After incubation for 3 hours at 27°C, the sludge was again centrifuged and an aliquot of the supernatant liquid was weighed after heating successively at 105°, 190° (to determine water of hydration), and 600°C. The loss of weight at 600°C reflects the amount of volatile organic matter given up by the sludge solids during the incubation. Amino acids and nucleotides in another aliquot of the solution were estimated by the method of Warburg and Christian (1941). The data are presented in Table 25.

Two significant points emerge from this experiment. First, more than 90% of the organic material in the supernatant liquid can be accounted for as amino acids and nucleotides. Determination of total Kjeldahl nitrogen in this solution showed a concentration of 32 μg/ml; this value represents 12% by weight of the amino acids and nucleotides as estimated spectrophotometrically, and it is in good agreement with the value of 14 to 15% that would be predicted. Ammonia, nitrate, and nitrite were not detectable.

Second, these results are consistent with the previous suggestion vis., that amino acids in the supernatant liquid from concentrated sludge are derived by leakage rather than by lysis. For, if the amino acids were derived by cell lysis, one would estimate that the amino acids and nucleotides would constitute roughly 60% of the total organic material, rather than 90% as was observed.

ENZYMES FROM FLOC FORMING ORGANISMS

Proteolytic Activity of the Sludge

One possible way of accounting for the foregoing observation that no soluble proteins can be detected in the sludge liquor would be the presence of an enzyme that rapidly digests the protein. To examine this possibility, a reaction mixture containing sludge at a final concentration of 2% solids and Bovine Serum albumin at a final concentration of 1.1 mg/ml was incubated for 3 hours at 23°C. The mixture was then centrifuged and the supernatant liquid divided into two aliquots. The amount of soluble tyrosine in one aliquot was determined after removing undigested protein with TCA. The total amount of tyrosine in the other aliquot was determined by incubating it for 2 hours at 23°C in the presence of 1 mg pronase/ml (a commercially available proteolytic enzyme) and then removing any undigested protein with TCA. The concentration of total tyrosine, measured after complete hydrolysis with pronase, was 60 μg/ml, a value that corresponds to 1.2 mg amino acid/ml. Hence, all of the Bovine Serum albumin added initially was recovered at the end of the experiment. The concentration of soluble tyrosine, however was 12 μg/ml, a value that corresponds to 0.24 mg amino acid/ml. Accordingly, only 20% of the added protein was hydrolyzed by the sludge in three hours. These values have been corrected for tyrosine liberated by the sludge under the same conditions of incubation, but without addition of exogenous protein.

This experiment suggests that proteolytic enzymes in sludge may not be very active, and that therefore there is a reasonable chance that the soluble amino acids of sludge are derived by leakage across the membranes of the bacteria. If so, sludge should not contain large amounts of extracellular protein. This appears to be the case. Thus, in several experiments, sludge containing different amounts of solid material was incubated at 23°C with pronase at 0.5-1.0 mg/ml. In every case, enzymatically treated sludge produced somewhat more tyrosine in these experiments, about 2/3 is soluble and about 1/3 is in an insoluble form that can be digested by pronase.

Enzymes from "Lytic" Cultures

The presence of "halos" around the colonies of bacteria isolated from sludge agar plates indicates the possible presence of matrix-degrading enzymes. These enzymes may be present either in insufficient amounts or they may be inhibited by substances in the sludge so that their activity is not obvious when sludge modification is attempted. By partially purifying and concentrating the crude enzyme mixture, matrix-digesting activity would be amplified.

Two cultures of the "lytic" organisms previously isolated on sludge agar were subjected to protein fractionation in an attempt to detect the presence of floc modifying enzymes. One hundred ml of "lytic" cultures SL-1 and SL-3 were inoculated into two liters each of autoclaved sludge and aerated by means of compressed air.

The next day the cultures were centrifuged and aliquots of the supernatant fluids were differentially salted out using 20, 40, 60, 80, and 100% saturation with dry crystalline $(NH_4)_2SO_4$. Precipitates were centrifuged out, suspended in minimal amounts of deionized water, and scanned in an ultraviolet spectrophotometer.

In none of the samples, including other attempts with other organisms, were peaks obtained that were unequivocally in the protein range (280 nm), which would have indicated the presence of an enzyme(s).

Another approach along these lines was to grow both "lytic" organisms SL-1 and SL-3 in nutrient broth and SL-1 alone in complete *Pseudomonas* medium. At 3 days, the cultures were centrifuged and the supernatants made to 70% saturation with $(NH_4)_2SO_4$. Water resuspended precipitates examined in the ultraviolet spectrophotometer showed peaks in three cases that were associated with the nucleic acid range (260 nm). It is very possible that the cultures were too old at the time they were salted out.

CONTROL OF FLOC FORMATION IN *ZOOGLOEA RAMIGERA*

Crabtree, *et al*. (1966) have shown that pure cultures of *Z. ramigera* I-16-M, grown in a liquid medium containing arginine as the sole source of carbon and nitrogen, remain dispersed throughout the growth cycle. When glucose is added to the culture at the end exponential phase, there is an immediate and striking increase in cell mass but not in cell number, and the cells form the typical flocculent aggregates.

If conditions are now altered by adding a source of nitrogen, such as NH_4Cl, the extracellular matrix seems to be digested. It thus appears that a culture of *Zoogloea* could be forced to produce a depolymerase by adding NH_4Cl after the matrix has started to accumulate. After NH_4Cl is added, the the flocs thereby dispersed, the culture can be centrifuged and the supernatant solution containing the suspected enzyme recovered. With this objective, an attempt was made to reproduce the work reported by Crabtree, *et al*.

Two ml of a 24-hour culture were added to each of three Klett sidearm flasks A, B, and C containing 50 ml of the basal arginine medium. The flasks were shaken continuously at 28 to 30°C during the experiment, and optical density readings were taken on each sample at 2 hr intervals.

Forty hours after inoculation, 0.5% glucose was added to all flasks. Flocculation occurred in all flasks. Eight hours later, the flocs in Flasks A and B were removed by centrifugation, washed once in phos-

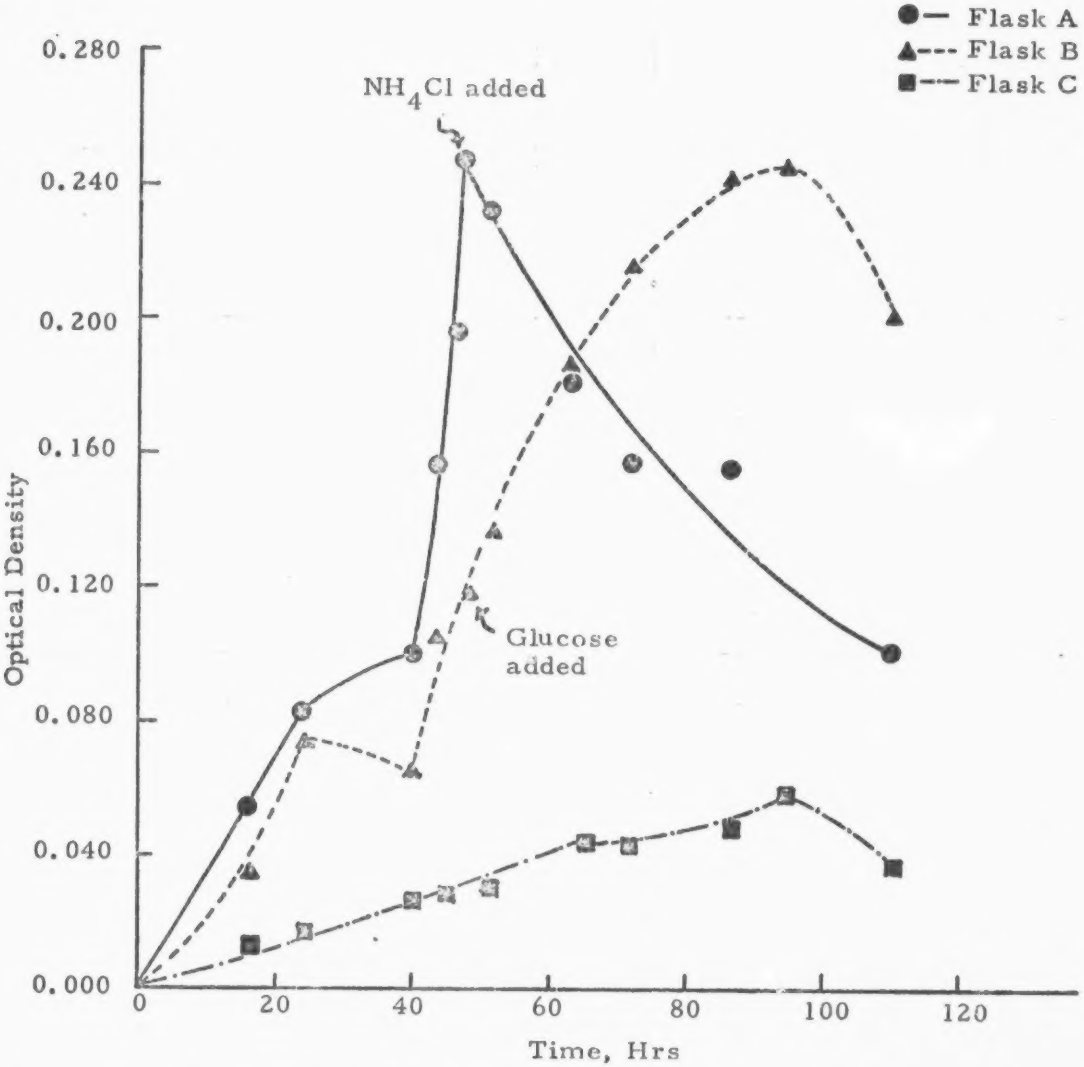


Figure 14. The Effect of NH_4Cl and Glucose on Zoogloea ramigera Strain I-16-M

phate buffer (pH 7.1), and then resuspended in 50 ml of the same buffer. To Flask A was added 0.1% NH_4Cl, and to Flask B, 0.5% glucose. Flask C was maintained as an untreated control.

Figure 14 indicates the response of the organisms to the experimental conditions. Within 16 hrs after inoculation into the basal medium, all flasks showed flocs with some dispersed growth, although Flask C did not flocculate as much as Flasks A and B. The dispersed growth, expected in the basal medium, failed to appear. The addition of glucose after 40 hrs markedly increased the amount of flocs and optical density in Flasks A and B, although Flask C was not appreciably affected.

The addition of NH_4Cl to the washed flocs of Flask A initiated a progressive decline in the optical density of the sample. Resuspension of the flocs of Flask B in the basal medium plus glucose resulted in a continued rise in optical density for 48 hours and a sharp decline thereafter. Flask C showed a gradual, but unremarkable, rise in optical density during the period of the experiment. The experiment was terminated at 112 hours when it became apparent that all flasks were being affected by the age of the cultures.

The sharp decline in optical density in Flask A after the addition of NH_4Cl was not accompanied by dispersal of the flocs; these persisted throughout the experiment.

Section VII

DISCUSSION

CHARACTERIZATION OF SLUDGE

There are numerous possible ways by which sludges may be characterized and compared. Suitable indices of sludge similarity would presumably include the enumeration of the primary microbial species, the specific activity of key enzymes, the rate of respiration in the presence and absence of key organic compounds, the type and quantity of fermentation end products, the concentration of specific immunogens, and the physico-chemical properties of the floc viewed as colloidal matter. Many of these characteristics of secondary sludges have been examined by numerous investigators, but there has not yet been a systematic correlation of the results among different sludges. For this reason, and because a detailed characterization of sludge was beyond the scope of the present research contract, examination was confined to a few characteristics that could be of importance in the enzyme studies. These include the Gram staining reaction of floc bacteria, the filtration and sedimentation rates of the sludge at pH 3 and at neutrality, the concentration of insoluble carbohydrate (determined by the anthrone procedure, which measures mono-, di-, and polysaccharides), the concentration of β-hydroxybutyric acid in the sludge, and the concentration of proteins, soluble amino acids, and other nitrogenous compounds (nucleotides, NH_3, NO_2^-, and NO_3^-) in the sludge liquid. As judged by these criteria, the sludge used in these studies were not greatly different from other sludges reported in the literature. On the other hand, the sludges examined in this study contained massive numbers of the anaerobic bacterium Spirochaeta plicatilis. These bacteria could not be accurately counted because many were tangled up in the flocs. However, judging from the fact that between 20 and 50 free spirochaetes could be seen in the field of an oil immersion lens, their number was probably in excess of 5×10^7/ml.

The appearance of an uncommon organism such as S. plicatilis in large numbers was at first difficult to explain. However, these organisms were later observed in the sludge of the Los Angeles Hyperion treatment plant. This plant uses the activated sludge process and receives the wastes from the city of Los Angeles. It is entirely separate from the Pomona and Whittier Narrows plants, which are part of the County system. The discovery of these spirochaetes in the sludge of the Hyperion treatment plant indicates that this organism may be more prevalent in sewage than published ecological studies would lead one to assume.

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This spirochaete is known to grow at the expense of amino acids in a micro-aerophilic environment, and these conditions apparently obtain in the secondary sludge at Pomona and Whittier Narrows. As will be discussed in more detail presently, soluble amino acids do occur in the sludge. Relative anaerobiosis in the sludge could be anticipated on theoretical grounds. For example, sludge probably consists mainly of strict aerobes belonging to the Gram negative Pseudomonadales, and at the actual concentration of 5 to 10 g bacterial dry weight/liter of sludge, it would be expected that most of the oxygen would be rapidly absorbed out of solution by this large mass of bacteria, even when the sludge is aerated. Some support for this can be gained by vigorously shaking a diluted sample of the sludge and then examining the suspension microscopically. The spirochaetes on the slide are completely immobile save in the liquid immediately surrounding the floc particles. With the passage of time, the zone of active mobility appears to extend outward from the floc, and it might prove interesting to study the rate at which this extension occurs.

The foregoing observations can be rationalized as follows. The spirochaetes are rendered immobile, but are not immediately killed, by the presence of oxygen. When the sludge is diluted, the flocs can not rapidly remove the oxygen introduced into solution by shaking, with the result that for a time the suspension is oxygen rich except in the liquid adjacent to the flocs where it is used up by the aerobic bacteria. If this interpretation of mobility is correct, S. plicatilis fulfills the function of an oxygen indicator in the sludge.

The presence of soluble amino acids and relative anaerobiosis in the sludges form an environment suitable for the growth of S. plicatilis, the same conditions in all probability exist in other sludges in which that organism has not been reported. In light of these observations, the Pomona and Whittier Narrows sludges were not considered typical. It would be of interest to survey sludges in various geographic areas to determine how widespread this organism really is.

The observation that even "well aerated" sludge is anaerobic has important implications from the viewpoint of enzymology. This anaerobic environment is undoubtedly a major factor in the stability of the waste sludge. Because the bacteria making up the sludge are primarily aerobes, they cannot metabolize in the absence of an oxygen supply and thus very limited amounts of proteolytic enzymes would be expected to be synthesized and appear in the medium. If the bacteria cannot produce proteolytic enzymes, then it follows that exogenously supplied enzymes (proteins) would have some finite lifetime in sludge liquor.

Reference to Figure 1 readily indicates that microorganisms in the floc are in fact embedded in a gelatinous matrix as opposed to a mere mechanical agglomeration of the cells.

A recent electron microscopic study of ultrathin sections of activated sludge floccules from industrial waste water that was examined under four different conditions (Bartsch, et al. 1970), revealed some interesting information that is repeated here. The flocs were uniformly colonized with bacteria, although only poorly in the case of developing flocs where extensive cell lysis occurred before the steady state had been achieved.

When stabilization of growth was achieved in the treatment plant the cells were thicker in the flocs and cocci appeared in addition to rods; the number of autolyzed cells was greatly reduced. In various flocs at this stage, the well formed matrix contained finely granular, thread-like structures. Numerous membranous elements also appeared in the matrix. The origin of these elements is not known, although it was suggested that they might arise from a fraction of the substrate; they were not part of autolyzed cells. Fimbriae were ruled out as the cause of the finely granular, thread-like structures.

When waste water was aerated and stirred rapidly, typical floc formation did not occur. Instead a rich mass of suspended single cells was obtained which were free of capsules and matrix material and contained cytoplasm indicative of cells in the logarithmic growth phase; hardly any autolyzed cells were seen.

In order to proceed with the present biochemical studies on the floc matrix, characterization of the sludge matrix was necessary. The action of various physical and chemical agents on the floc material were investigated in order to elucidate the mechanisms involved in maintaining floc integrity.

Here it may be pertinent to ask the question "what per cent of the floccules would have to be affected by any treatment - physical, chemical, or enzymatic - before the effects could be appreciated when sludge was examined in the microscope?" While no answer can be offered to this valid question, one is tempted to say subjectively that the percentage would have to be high in order for it to be detected. The only way, other than perhaps viewing freed bacteria, that one could tell whether or not floccules were being attacked would be to observe some unequivocal morphological stage, intermediate between intact floccules and freed bacteria, resulting from attack. Such a stage has not been determined with certainty in these studies.

Although admittedly the application of physical and chemical agents to sludge cannot be considered as biological sludge dewatering, nonetheless it was felt that if these agents did appreciably cause deflocculation, this could give information as to the kinds of biological mechanisms that should be investigated.

Heat and urea, either alone or in combination, are well known for their ability to affect stability of polymers, as are the various gelling materials used in this program. In the experiments reported here there was little, if any, value obtained through the use of gels.

Urea by itself at room temperature was not effective in the dissolution of flocs; it could, however, have a useful place in conjunction with agents whose chemical activity might complement that of urea. It would appear that heating does free floccule bacteria, and that hot urea frees more bacteria from floccules than cold urea. Whether this action can be employed to advantage has yet to be determined.

EFFECT OF BUFFERS AND SALTS ON THE FILTRATION RATE OF SLUDGE

These experiments showed that the common buffers used in enzymological work, with the exception of citrate, did not alter the filtration rate of sludge at any pH tested, in comparison with sludge samples in which the pH was adjusted with HCl or NaOH in the absence of exogenous buffers. Furthermore, the addition of Mg^{++}, a cofactor for several of the enzymes used, did not in itself change the filtration rate. Accordingly, it was possible to treat the sludge with enzymes under standard conditions used in enzymological work.

The rate at which sludge could be filtered showed an interesting variation with respect to pH. At pH 3, the filtration rate was strikingly rapid. As the pH was increased, the filtration rate became slower. There was some evidence that at pH values greater than 3, the rate of filtration was inversely proportional to the pH. However, this effect was not at all pronounced, and in general it appeared that the sludge filtered slowly at every pH value tested except at 3.

The rapid filterability solely at pH 3 is difficult to rationalize. Since this is the isoelectric point of many bacteria (Dyar and Ordal, 1946; Dyar, 1948), it would appear that the most rapid rate of filtration occurs when the flocs carry no net electrical charge. From the well-known behavior of such amphoteric compounds as protein, it would be expected that the filtration rate of sludge would decrease gradually as the flocs are given an increasing negative charge by raising the pH. The fact that the filtration rate increases only at the presumptive isoelectric point of the floc suggests that the sludge dewaters at some relatively slow characteristic rate as long as any net charge remains on the flocs. The reason for this behavior is obscure.

From an experimental outlook it was considered that some of the selected enzymes might remove acidic or basic functional groups from the sludge floc and thereby alter the isoelectric point. Since alteration of floc characteristics, rather than complete hydrolysis, is one aim of the enzymological work, the sludge was incubated with each enzyme at the pH for its optimal hydrolytic activity, as indicated in the tables, and then filtered at different pH levels in order to determine whether or not the isoelectric point had been changed by enzymatic digestion.

In the presence of citrate buffer, the rate of filtration showed a definite inverse proportionality to pH. From the preceding observations, this effect could not be attributed to changes in the net charge of the flocs. Rather, it was ascribed to the increasing charge of the citrate molecule itself, a notion which suggested that the "citrate effect" is due to chelation of some ion that is essential for the normal filterability of untreated sludge. The actual ion involved was not identified. However, the observation was made that when the sludge was incubated with citrate and excess Mg^{++}, the filtration rate was considerably faster than that of control mixtures without Mg^{++}. Furthermore, analysis of the filtrate showed that citrate caused the sludge to release iron, but that Mg^{++} was able to reduce the amount of iron in the filtrate as well as to increase the filtration rate of sludge buffered with citrate. These results suggested that iron might be the metal actually involved in the "citrate effect." This possibility was strengthened by the fact that α, α-dipyridyl (which specifically complexes ferrous ion) also reduced the filtration rate. Ferric ion may play only a minor role in enhancing the natural filterability of sludge, for when sludge was incubated with Tiron, a reagent that complexes ferric ion, the filtration rate was unchanged. These experiments suggest that ferrous ion may play a significant role in determining the rapidity of filtration of a given sludge in the absence of other additives. It is possible that variations in the filtration rate of different sludges are associated with the concentration of Fe^{++} in the raw sewage, as well as with the mass of the sludge which, by its effect on the concentration of dissolved oxygen, would to some extent determine whether or not the ferrous ions would spontaneously auto-oxidize to ferric ions.

MASKING OF BIOLOGICAL MODIFICATION

During the initial tests, when filtration rates proved to be so slow in samples taken from the flasks containing antibiotics, the rationale of dewatering testing in these experiments was questioned. The assumption had been made all along that the faster the filtration rate, the more efficient the dewatering process and the lower the water holding capacity of the floc matrix. Undoubtedly this holds true in most cases. There is, however, an alternative possibility, namely, that when filtration is slow, the very slowness may be due to the large number of bacteria that have been freed from sludge floccules, as a result of floccule dissolution brought on by the various treatments and consequent clogging of the filter paper by the free bacteria. Clogging of filters by dispersed bacteria is a phenomenon well known to bacteriologists. The end result of this speculation is the proposed interpretation that slowness of filtration reflects a biological alteration of the floc matrix that could be used to advantage when applied on a larger scale. An indication that something had been released from the flocs into the supernatants was the observation that when the sludge in the above flasks had had an opportunity to settle in an undisturbed manner, the supernatant fluids of the antibiotic-containing flasks were turbid, whereas the control supernatant was clear. Also, the filtrates from the dewatering tests were turbid in the antibiotic samples and clear in the control. A second possibility that may account for poor dewatering is the release of viscous by-products into the supernatant fluids as a result of treatment. This would mask any improvements in the dewatering characteristics of sludge. Under the possibilities described here it was likely that processes that showed promise for altering matrix material were actually being over-looked as a consequence of the screening procedures.

DEWATERING BY MEANS OF ENZYMES

As was brought out in the section on Sludge Modification, there was a dramatic increase in dewatering rate when sludge samples were treated either with lysozyme-streptomycin combinations or lysozyme alone (Tables 10, 15, 16, 17). In both types of experiments, the filtration was so rapid that the test equipment could not measure the initial rates needed for calculating

$$\frac{\theta}{V^2} \text{ (rate) values.}$$

Since the speed of filtration was at a maximum for the equipment used, further improvements in dewatering would be undetected and consequently the values reported are conservative. Sludges with higher per cents solids should respond in a more favorable manner. In the supernatant replacement technique no further improvement could be achieved in the treated samples because of the insensitivity of the equipment to additional increases in dewatering. However, the control did improve, thus narrowing the contrast between it and the experimental samples and resulting in a lower control/experimental filtration ratio.

The apparent ineffectiveness of streptomycin under anaerobic conditions could be explained on the basis of the work of Kogut, et al. (1965). These workers investigated the frequently reported observation that streptomycin is ineffective against strictly anaerobic or facultively anaerobic bacteria when they are grown under anaerobic conditions. Their studies used Escherichia coli B, which proved to be insensitive to streptomycin when growing exponentially under anaerobic conditions. It was concluded that the uptake of the antibiotic into the cells was prevented by anaerobic conditions, whereas aerobic conditions allowed streptomycin to be taken up by the cells with consequent expression of antibiotic inhibition.

Randall, et al. (1971) reported on the effect of enzyme activity in the sludge as related to water retention. They demonstrated that water retention in the sludge decreased as the enzyme activity decreased.

In the present studies, there were two major circumstances under which rapid dewatering manifested itself. One was an aerobic environment in the presence of streptomycin and the other was an anaerobic environment without the antibiotic requirement. In both cases a large amount of lysozyme was also needed, although the optimal amount of this enzyme has not been determined.

Actually these two apparently diverse alternatives for achieving rapid dewatering may be bringing about similar changes in the sludge environment. The fact that streptomycin inhibits protein synthesis and alters cell metabolism is well accepted. If the sludge microorganisms are considered to be predominantly obligate aerobes, the imposition of anaerobiosis would have an effect similar to that produced by streptomycin in that the cell metabolism would be drastically changed. Alteration in cellular physiology brought about by either of these conditions appears to be a requirement for the action of the lysozyme.

Several mechanisms may be postulated to account for lysozyme activity. One of the simplest of these would be that the lysozyme acts directly on the sludge matrix. Anaerobiosis may prevent the synthesis of either additional matrix material, or lysozyme-destroying enzymes.

Another very likely possibility is that the matrix depolymerase is already present within the microbial cells of the floccules and it is released through modification of the cell wall by lysozyme. Anaerobiosis in this case may either prevent repair of cell wall damage or inhibit the synthesis of an enzyme repressor essential to metabolic regulation in the whole organism.

Combinations of antibiotics with lysozyme have been tested by other workers to determine if synergistic action results from combining antibiotics with this enzyme. For example, Ritzerfeld (1969) reported that lysozyme enhanced the antibacterial effect of penicillin and chloramphenicol against *Staphylococci*, but not against *Escherichia coli*; Nagai and Hashmoto (1969) found that lysozyme together with colistin was active in inhibiting the growth of *Staphylococcus aureus*, even when this organism was resistant to colistin alone; Nakazawa, *et al.* (1968) have shown that lysozyme and aminobenzylpenicillin are together synergistic in their action against several Gram-positive and Gram-negative bacteria, and they recorded significant inhibition of *E. coli* when lysozyme was added at the initial stage of the culture and aminobenzylpenicillin at the logarithmic stage.

Through the application of classical biological techniques, the general mechanism responsible for floc modification can be ascertained. An understanding of the processes involved would lead to investigation of alternative methods of treatment or selection of additional ways of controlling the conditions necessary for enzyme action.

PROTEOLYTIC ACTIVITY OF SLUDGE

The sludge contains small amounts of proteolytic enzyme(s) as shown by its ability to hydrolyze exogenously added Bovine Serum Albumin. The rate of hydrolysis, however, is very slow, with only 20% of the substrate being hydrolyzed to amino acids in the course of three hours at 23°C by concentrated sludge. These results can be attributed to low enzymatic activity rather than to low substrate concentration, as would occur were the substrate largely adsorbed to solid matter in the sludge, for all of the amino acids initially added as albumin were recoverable in the sludge liquid. From these experiments it was concluded that enzymes could be added to the sludge without risk of rapid inactivation by sludge protease activity.

In characterizing the sludge environment it was observed that fresh sludge with a dry weight of 5 g/l, contained soluble material that reacts with ninhydrin and other reagents used to detect amino acids. This material was equivalent to 60 mg amino acid/l, and it was not precipitated by 10% trichloroacetic acid. Since about half of the solid matter in sludge is protein, as determined by the method of Lowry et al. (1951), it can be readily calculated that this material represents nearly 3% of the total amino acid content of the bacteria in the sludge.

In order to identify the unknown material by chromatography, concentration of the sludge liquid was necessary. However, when flash evaporation was used for this purpose, most of the material was lost, possibly by adsorption to a copious inorganic precipitate that formed as the solution was concentrated. As an alternative approach, the sludge itself was concentrated to 50 g dry weight/l by centrifugation and resuspended in a little of the supernatant liquid. After brief incubation, the suspension was centrifuged again, and the supernatant solution was chromatographed on paper. All of the common amino acids, with the possible exception of arginine, appeared to be present in the solution. Their concentration, as estimated by several methods, was about 600 mg/l, an amount equivalent to 3% of the total amino acids available in the concentrated sludge. During prolonged incubation, the soluble amino acids increased at a rate equivalent to 0.3% of the total bacterial amino acid content per hour.

These observations strongly suggest that the unknown material noted in the dilute sludge does, in fact, consist of amino acids. This suggestion is strengthened by the fact that other workers have also detected amino acids in sludge, although at a somewhat higher concentration than that found in the Pomona sludge (Teletzke, et al., 1967). It is possible that Pomona sludge has an abnormally low content of free amino acids owing to the growth of *S. plicatilis* at their expense.

Soluble amino acids in the sludge could be derived from cellular autolysis followed by rapid protein hydrolysis. At least two arguments can be advanced against this hypothesis. First, gravimetric analysis shows that 90% of the soluble organic matter in the liquid phase of sludge can be accounted for as amino acids and nucleotides. This value is nearly twice that expected for a general cellular autolysate. Second, judging by the inability of 10% trichloroacetic acid to precipitate soluble organic matter, the liquor contains little protein or nucleic acid. The absence of protein is not consistent with an autolytic origin of the amino acids, for in view of the low proteolytic activity already mentioned, free protein should persist for a considerable time following cell lysis. The possibility that proteinaceous components of a cellular autolysate are adsorbed and digested on surfaces in the sludge was not examined directly, however, it is an improbable explanation for the absence of soluble protein, since only partial or negligible adsorption occurred when certain proteins (e.g., poly-β-hydroxybutyric acid depolymerase and Bovine Serum Albumin) were added exogenously to the concentrated sludge. It is assumed that these arguments also apply to the relatively small quantity of soluble nucleotides, but this point cannot be considered proven until the nucleotides have been identified and until nuclease activity in the sludge has been studied.

If, as it appears, cellular autolysis plays an insignificant role in the sludge samples that have been studied, then the soluble amino acids and nucleotides must be derived by leakage across the cell membranes of the microbial population. The physiological basis for this event can be partially attributed to the relative anaerobiosis of the sludge. The evidence for anaerobiosis rests on three points: (1) the growth and motility of S. plicatilis; (2) the possible absence of soluble arginine, which is the only amino acid that Pseudomonas species can use for energy in the absence of air; (3) the fact that the sludge liquid is colorless but turns yellow after the sludge solids are removed from it by centrifugation. This phenomenon occurs because sludge contains an unidentified soluble pigment that has a single absorption peak at 430 nm when oxidized by air or ferricyanide, but that does not absorb light when reduced with dithionite (Schinazi, 1969).

Under anaerobic conditions, a population composed largely of strict aerobes should be unable to synthesize sufficient ATP to meet its major energy requirements. The bacteria of sludge are probably in the stationary phase of growth, and at this stage the principal energy-demanding reactions are associated with protein and nucleic acid turnover involving an exchange of protein amino acids with the amino acid pools (Mandelstam, 1963). As oxygen becomes limiting, due to cell metabolism, the rate at which these macromolecules are degraded should exceed the rate of synthesis, and accordingly amino acids and nucleotides should accumulate in the intracellular pools. Since the internal pools are capable of only limited expansion, these small molecules should soon begin to leak from the cells (Cowie and McClure, 1959; Britten and McClure, 1962). If this interpretation is correct, it would account for the fact that the actual amount of soluble amino acids and nucleotides in the sludge liquid is roughly ten-fold in excess of the intracellular pools. The lack of ATP prevents the reassimilation of the amino acids and protein breakdown may thus proceed to the extent that the cell is damaged beyond the point of recovery. Eventually death and complete lysis should follow (Marr, et al., 1963).

Without studying the life history of sludge in detail, it is impossible to specify the phase of growth occupied by the bacteria at a given time. The paucity of nutrients in raw sewage could not conceivably support more than a small increase in cell numbers, considering the massive inoculum of sludge used at Pomona (Wuhrmann, 1964). Furthermore, Gaudy has shown that even when a nutrient rich medium is inoculated with sludge, the bacteria do not begin to grow exponentially for long periods of time (Rao and Gaudy, 1966). From these observations, it is assumed that sludge bacteria are in the late stationary growth, and possibly on the verge of irreversible cell damage. If correct, this notion has two important implications. First, it suggests that a large proportion of the population is non-viable as indicated by other observations that viable counts of sludge bacteria are less than direct counts (Unz and Dondero, 1967). Second, it suggests that owing to the large mass of oxygen-consuming bacteria, aeration has at best very little effect on cell viability. If so, experiments in which aerated and unaerated sludges have been compared in the past might have very little bearing on the actual state of the sludge, since in both cases the cultures are actually anaerobic and no physiological differences should exist (Haseltine, 1961).

Of more significance to the current program is the observation that the physiological state of the sludge prevents it from endogenously forming the enzymes required to mobilize its internal food reserves once external nutrients have been depleted. Thus storage products such as poly-β-hydroxybutyric acid and possibly other polymeric forms will remain associated with the floc bacteria and presumably continue to exert their physico-chemical effects. Thus, without external intervention the sludge properties will change very slowly. A promising aspect of this is that organic additives such as enzymes should be able to survive and function in this environment.

EFFECT OF PHBA-DEPOLYMERASE AND OTHER ENZYMES

PHBA-depolymerase, isolated from the culture medium after the growth of Pseudomonas lemoigenei, rapidly digested purified poly-β-hydroxybutyric acid polymer granules in the presence of sludge. When the sludge was incubated with depolymerase alone, however, no solubilization of hydroxybutyryl residues could be detected during incubation periods up to sixteen hours in length. At this time all of the enzyme initially added would have been destroyed by the natural proteolytic activity of sludge, and further incubation was therefore not attempted. Addition of the depolymerase to sludge failed to digest the endogenous polymer. Such treatment also had no effect on the filtration rate in comparison with sludge from which the depolymerase was omitted.

Similar results were obtained with sludge that was incubated with trypsin before adding the depolymerase, in order to solubilize any protein that might be adsorbed to endogenous polymer so as to block access of the depolymerase to its substrate. Finally, no dispersal of the floc was observed in any of these experiments.

These observations add no support to the theory of Crabtree, *et al.* (1965) (1966) but neither do they disprove it. The theory is, in fact, difficult to test, for no specific physico-chemical mechanism relating synthesis of intracellular poly-β-hydroxybutyric acid to floc formation was suggested. Crabtree, *et al.* describe three possible mechanisms to account for the observation that cells of *Z. ramigera* clump together only when they contain microscopically visible granules of the endogenous polymer:

(1) Some of the cells autolyze, and the polymer granules which are then released into the medium serve as centers for adsorption of the remaining intact cells. This suggestion, insofar as it applies in sludge, is probably incorrect, for not only was the depolymerase unable to affect the floc but also no evidence for cellular autolysis could be obtained.

(2) When the cells accumulate massive quantities of intracellular polymer, the cell wall is altered in such a way that it becomes adhesive so that the cells stick together. In pure cultures of *Z. ramigera*, the nascent flocs do appear to contain closely opposed cells. In sludge, however, the individual cells of the floc are quite widely separated and without Brownian motion, as though they were embedded in a jelly. A further argument against the second postulate is that the sludge contains only small amounts of poly-β-hydroxybutyric acid, so that the massive accumulation of polymer required in this hypothesis does not appear to occur in sludge.

(3) The cells are moored to each other by intercellular strands of β-hydroxybutyryl moieties. None of the present observations argue directly against this postulate, especially as it is not known if the depolymerase can hydrolyze hydroxybutyryl esters in such a configuration.

The experiments reported here indicate that synthesis of poly-β-hydroxybutyric acid and floc formation may not be casually related. The old idea that flocs are composed of bacteria embedded in an extracellular matrix is supported by our microscopic observations, and recent workers have provided evidence that the matrix is composed of polysaccharide (Finstein, 1967; Friedman, *et al.* 1968). This polysaccharide could be an extracellular reserve material, as well as the cause of flocculation. If so, its synthesis should be regulated by the same environmental factors that affect the accumulation or dispersal of all reserve materials, including poly-β-hydroxybutyrate. Accordingly, synthesis of the polysaccharide and of poly-β-hydroxybutyrate should be strictly coordinated with floc formation, and floc dispersal should coincide with degradation of the polymers. In the light of this idea, poly-β-hydroxybutyric acid does not cause floc formation, but it is an indicator of the synthesis of a polymer that leads to flocculation of Z. ramigera.

The question of the involvement of PHBA acid is discussed further in a report by Angelbeck and Kirsch (1969). These workers examined two strains of Z. ramigera, ZRC and 116M, which did not reveal capsules by means of capsular stains, although a personal communication to the authors from Dugan reported that a polysaccharide microcapsule could be seen in strain 116M by means of the electron microscope.

The pH of the growth medium, the presence of Ca^{++} and Mg^{++}, and the age of the culture greatly influenced the occurrence of aggregation of this bacterium. For example, cells preconditioned by growth at pH 7 did not aggregate at pH 6, and vice versa. Calcium and Mg^{++} ions prevented aggregation in pH 6.0 growth medium and 10 times more Mg^{++} than Ca^{++} were required for the same dispersion of cells. The authors state that "the PHB content of both aggregated and non-aggregated cells was found to be approximately 1% of the cellular dry weight. It was concluded that there was no compelling reason to associate the PHB accumulation with the aggregative mode of growth which is induced by the organisms at a depressed pH."

A series of enzymes capable of degrading other intracellular bacterial polymers were tested concurrently with the PHBA depolymerase. Two enzymes specific for cell wall components were included in this group along with the two nucleases. These latter were tried because the physical properties of nucleic acids are such that they could account for the gumminess of sludge. No change in filtration rates was observed when waste sludge was treated with poly-β-hydroxybutyric acid depolymerase, α-amylase, β-amylase, cellulase, chitinase, DNAase, lipase, lysozyme peptidase, pronase, RNAase, or trypsin. These negative results reinforce the view that the polymer of the matrix is not an intracellular material released into the medium through the mechanism of cell lysis.

That enzymes capable of dissolving the polymers that make up the floc matrix do exist may be presumed from the work of Crabtree, et al. (1966). They showed that typical flocs of Z. ramigera could be made to disperse under appropriate nutrient conditions. Because the existing matrix must somehow be involved, it must be assumed that the bacteria are able to produce the appropriate depolymerizing enzyme. If the composition of the matrix is complex, it would be expected that several enzymes are required to bring about its dissolution.

As indicated in Figure 6, attempts to duplicate the work of Crabtree, et al. with Z. ramigera I-16-M were unsuccessful. The culture would not grow in a totally dispersed phase in the minimal medium. Although added glucose caused increased flocculation of the cells, the addition of NH_4Cl to a washed suspension did not result in the expected rapid deflocculation. Apparently, control of the composition of the medium is not the sole requirement for floc dispersal. This does not obviate the presence of a depolymerizing enzyme in this organism, but does evoke the necessity for a better definition of the conditions under which it is expressed.

MATRIX EXTRACTION

As indicated in the Experimental Section, only about 15% of the sludge was alkaline extractable. Subsequent extractions produced additional material, but this was complicated by the gelatinous nature of the previously extracted sludge. It is probable that the sludge matrix is chemically heterogeneous, being made up of more than one polymer, so that only part of it exists in an alkali soluble state. Thus if only a single depolymerizing agent were developed, a maximal amount of 15% of matrix material would be altered.

A greater possibility of obtaining floc dissolution would exist if the sludge microorganisms themselves could be induced to form the necessary depolymerizing enzymes. This could be accomplished either by supplementing the sludge with essential nutrients to stimulate the growth of the dormant floc microorganisms, or by isolating specific organisms capable of utilizing matrix material.

Anderson (1964) reported that "chromatographic analyses of the hydrolyzed polysaccharide extracts of floc matrix revealed glucosamine and arabinose." His results were obtained from municipal and domestic sources from which he isolated 70-73% gram negative strains from the flocs. Zoogloea predominated, and the implication is made that members of this genus are responsible for floc formation, and by extension for the floc matrix, which is composed of polymers of an hexosamine and a pentose monosaccharide. This, of course, is in marked contrast to the two monosaccharides (glucose and galactose) reported as the building blocks of Z. ramigera matrix (Parsons & Dugan, 1971).

These latter authors isolated Z. ramigera polymer by NH_4OH extraction, hydrolyzed it in mineral acid, and found that it contained only glucose and galactose, with glucose as the predominant sugar. The disaccharide formed from glucose and galactose is lactose, the configuration about the bond being a β-linkage.

As indicated in the section on Sludge Modification, the one attempt here to use β-galactosidase, which attacks β-linkages, did not result in improvement in the dewatering of sludge. This may have been due either to the absence of the substrate or because the chemical bonds were not readily available to the enzyme. Dugan was a co-author on an earlier paper (Friedman, et al., 1968) in which it was claimed that hydrolysis of the isolated polysaccharide polymer of Z. ramigera yielded only glucose as "the primary and possibly the only constituent of the polymer". It was suggested that since impure cellulase acted upon the polymer it resembled cellulose and probably contained 1,4-β-glycosidic linkages.

This latter observation is of considerable interest since lysozyme acts by hydrolyzing polysaccharide in the bacterial cell wall, by breaking the β-1,4 bond linking together N-methylmuramic acid and N-methylglucosamine. In view of the dewatering obtained through the use of the enzyme lysozyme, the significance of the presence of these chemical linkages, apart from the microbial cell wall, cannot be dismissed.

SLUDGE AS A MEDIUM

As presented in the Sludge Characterization Section, several microorganisms were isolated on sludge agar plates. The organisms of interest were all characterized by the ability to form "halos" in the agar surrounding the colonies.

The addition of organic nitrogen to the sludge agar, in the form of 0.1% peptone, tryptone, or Casamino acids, resulted in faster growing colonies of "lytic" bacteria, with both larger colonies and more prominent "lytic" zones around the colonies. This sort of stimulation by organic nitrogen may lend itself to further useful experiments.

It was most often seen, during examination of sludge agar plates that had been spread from a complex source, such as sludge itself, that several obviously different kinds of colonies were exhibiting "lysis" on the same agar plate. Since it has been a cardinal consideration during these studies that dewatering might well result from the combined action of several microorganisms, rather than from the action of one microorganism alone, it was of some interest to try and see if several such organisms growing together would enhance the lytic phenomenon. To do this, parallel and intersecting streaks of growth were made of the various lytic colonies on fresh sludge agar plates.

Whereas inhibition of growth could be detected on occasion where two streaks of growth intersected, no instances of enhanced lysis were ever observed, although the normal amount of lysis was apparent along each streak. Thus, none of the lytic organisms tested appeared to reinforce each other. This could be interpreted to mean that the same kind of enzyme(s) is responsible for the "lysis" regardless of the species producing it.

The suspicion was entertained that the "lytic" zone around a colony might simply be a function of accumulated metabolic waste products, mainly organic acids, with a resultant localized low pH and acid hydrolysis of the sludge components. However, when pH papers of different ranges were laid across the sludge agar and over colonies, there was no indication of an acidic pH response, although the sensitivity of such a test admittedly leaves much to be desired.

A further investigation was carried out by cutting out wells with a cork borer, in both regular sludge agar plates and in sludge agar buffered with phosphate to neutrality, and then filling the wells with known buffers of pH's 2, 4, 6, 8, and 10. Observations showed that clear zones appeared in the sludge agar around the wells containing buffer at pH 2 and that there was very slight clearing around the wells at pH 4. No haloing effect was apparent around the wells at higher pH's. It was the general consensus that the pH 2 clearing did not appear like the clearing around colonies, in that it was too clear and sharply delimited.

The lytic phenomenon is of great interest in light of the results obtained with the enzyme lysozyme. It was naturally expected that this "lytic" property of the microorganisms could be exploited for sludge dewatering.

It appears that the "lysis" of the sludge is the result of the excretion of an enzyme(s) that causes lysis of the microbial cells embedded in the sludge agar, and perhaps of the sludge matrix as well. The zones of lysis occurred with great regularity around the colonies spread from a known "lytic" culture and the ultimate size of the zone depended upon the age of the colonies, i.e., the zone was smallest around young colonies and widest around older colonies. In other words, the zone was a collective product of the age of the organisms comprising a colony.

Although a number of different species of microorganisms has been isolated that exhibit the phenomenon of lysis on sludge agar plates, it was noted that the most commonly occurring of these organisms were aerobic, Gram-positive, spore-forming rods that formed colonies typical of members of the genus Bacillus. Moreover, identified pure cultures of Bacilli were shown to produce similar clearings on sludge agar to the "wild" or sludge-isolated strains mentioned above. It will be recalled from the Experimental Section that the optimal growth temperature for the pure cultures of Bacilli did not necessarily coincide with the temperature that produced the most extensive or the fastest appearing lysis.

These observations are interesting in the light of the work of Nomura and Hosoda (1956), Richmond (1957), and Kruf and Smekal (1969) on autolytic enzymes, or bacterial "lysozymes", in members of the genus Bacillus. In working with amylase-producing strains of B. subtilis, Nomura found that when the cultures were grown aerobically in shaking cultures and then left without further aeration, the phenomenon of "anaerobic lysis" occurred, i.e., very rapid autolysis under anaerobic conditions. Later, Nomura and Hosoda (1956) isolated "autolysin", the autolytic enzyme, by means of $(NH_4)_2SO_4$ precipitation, and found it to be non-dialyzable with an optimum pH range of 6 to 8. The primary action of the autolysin is on the cell wall, probably on the polysaccharide contained in it, after the manner of the action of egg-white lysozyme.

Richmond (1957) isolated a Bacillus from soil which produced a lytic factor in the course of exponential growth. The biological properties of the lytic enzyme were so similar to those of egg-white lysozyme that Richmond called it a bacterial lysozyme.

Kruf and Smekal (1969) have grown B. subtilis BL-750 in submerged culture and shown that large amounts of a lytic enzyme, apparently identical to bacterial muramidase (lysozyme), could be obtained.

All of the work involving the lytic enzymes of the Bacilli would seem further to indicate that these organisms may be exploited as a cheap source of bacterial lysozyme in the dewatering process reported here. Bacilli grow readily on sludge agar and in autoclaved sludge and it should be relatively easy to determine the optimal length of time required for the maximal production of their lytic enzyme(s).

The inoculation of pure cultures of "lytic" bacteria into flasks of autoclaved liquid sludge resulted in extensive growth of these bacteria, although the flocs appeared unchanged when viewed microscopically, even after several successive passages in autoclaved sludge. Unfortunately, the technique of supernatant replacement had not been developed at this stage of the studies and much of this work should be repeated making use of this technique.

ENGINEERING CONSIDERATIONS

The ratio $$\frac{\theta}{V^2}$$

used in presenting the experimental data in this report is a measure of the rate of filtration of the various sludge samples. This rate can be used to calculate the reduction in volume that can be achieved in a given sludge during vacuum filtration over a period of time. The information presented in Tables 18 and 19 for the two cases analyzed shows that roughly a 10 to 30 fold increase in solids can be achieved over a 5 minute period. The final sludge moisture varies from 73% to 93% and thus the liquid volume reductions indicated in the tables represents a significant economy in liquid handling in a treatment plant. Moreover the filtrates from the experimental samples in Table 19 when analyzed for solids and turbidity agree with the visual observation that a clear effluent is produced after enzyme treatment.

Another important aspect in evaluating changes in filtration characteristics is to determine the point where it may become uneconomical to further reduce the moisture content. Figure 10 illustrates this for the data presented in Table 18. In this experiment there occurs a change from a fairly rapid dewatering rate to a slower rate at about 92% moisture content. However, in the experiment presented in Figure 19, there is a precipitous increase in dewatering rate which in part may be due to the higher solids content of the starting sludge. Speculation may be made that this rate change occurs when the filter cake reaches a critical thickness, providing an optimally structured filter bed for further dewatering. It is obvious that the effects of starting sludge solids concentration should be one of the prime areas of investigation in an extension of this work.

A method that is universally applicable for characterizing different sludges is the determination of the specific filtration resistance (r) of the sludge. This determination allows one to evaluate the behavior of sludges under defined conditions and to predict their behavior in various processing equipment. Specific resistance is a true measure of the filterability of a sample, and thus it is possible to compare samples numerically.

The specific resistance has been calculated for two sets of experiments using the lysozyme and streptomycin-lysozyme combinations as presented in Table 20. This parameter is an important factor in predicting the yield of solids to be expected from the processing equipment. By knowing the specific resistance, calculations could be made for pressure filters, vacuum filters, and for other types of plant equipment.

Based on the information provided in Table 20, the vacuum filter yield of dry cake solids from 1000 gallons of 0.73% initial solids sludge would be 609 lbs. and would require 1 hr. for the filtration. The 0.67% sludge would yield 559 lbs. of solids from 1000 gals., requiring 0.77 hr. of filtering time.

The lysozyme process compares favorably with other sludge pretreatments and has distinct advantages as far as disposability of the final product is concerned. However of greater importance is the fact that an entirely new approach to sludge disposal has been revealed.

The technical feasibility of directly altering the water rataining properties of the matrix materials has been demonstrated. This method utilizes the relatively mild conditions inherent in a biological system. Further advantage should be taken of this unique process by:

1. developing the necessary parametric information for a pilot plant process.

2. undertaking a program to reduce process costs, including a study of a cheaper source of enzymes.

3. pursuing a search for additional enzymes or chemical-physical processes that may simulate the activity of the lysozyme.

SECTION VIII

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SECTION IX

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SECTION X

APPENDICES

A. SELECTED ANALYTICAL PROCEDURES

1. Filterability

The sludge was filtered in 10 ml aliquots under a partial vacuum of 26 cm Hg through Whatman #1 filter paper clamped in a cylindrical Millipore filter chamber of 20 mm diameter. The filtrate was collected in graduated centrifuge tubes placed in the vacuum flask beneath this apparatus. The filterability was calculated in some cases as specific resistance, r, according to the following equation:

$$r = \frac{2bPA^2}{\mu C}$$

where P = pressure (g/cm^2), A = filter area (cm^2), μ = filtrate viscosity (poises), C = solids content (g/ml), and b is the ratio of θ/V to V, where V = filtrate volume (cm^3) at time (seconds) (Fair and Geyer, 1958; Burd, 1968). In most cases, however, the filtration rate was calculated as $b = \theta/V^2$, where b is the slope obtained by plotting θ/V as a function of v.

2. Calculation of Yields from a Vacuum Filter

The yield on a vacuum filter may be expressed by the following equation:

$$L = 0.0357 \frac{(100-C_f)}{(C_i - C_f)} \quad \frac{m\,PC_i \quad (100-C_i)}{\theta R \mu}^{1/2}$$

where

L = yield of dry cake solids, lb/sq ft/hr

C_i = initial moisture content of the sludge, %

C_f = final moisture content of the sludge, %

m = proportion of the time for which suction acts, %

θ = time for one drum revolution, min

P = the average suction pressure, psi

μ = the viscosity of the filtrate, centipoises

R = $r/10^7 \ sec^2/g$

3. Magnesium and calcium were determined by titration with EDTA (Welcher, 1958).

4. Iron was determined by the phenanthrolene method after reduction to Fe^{++} by boiling with acid and hydroxylamine (Manual of Indust. Water & Indust. Waste Water, 1965).

5. Soluble β-hydroxybutyryl compounds were determined by the colorimetric method of Slepecky and Law (1960). Poly-β-hydroxybutyric acid was dissolved in chloroform, and aliquots of the solution were measured for β-hydroxybutyrate by the same method.

6. Carbohydrate was estimated with the anthrone reagent, using glucose as a standard (Hassid and Abraham, 1957).

7. The β-hydroxybutyrate and carbohydrate in sludge were measured by the methods just described after subjecting the sludge to a variety of treatments and extraction procedures. The following procedure was used routinely for determining the β-hydroxybutyrate and carbohydrate contents of sludge.

A sample of sludge is centrifuged, the supernatant liquid removed and the pellet resuspended in distilled water. To the pellet fraction sufficient concentrated HCl is added to make the final solution 1 N. This suspension is then heated under pressure to 120°C for 20 minutes (autoclaved). After the solution is cooled to room temperature, it is centrifuged at 20, 000 G for 10 minutes, the supernatant saved, and the pellet resuspended in distilled water up to its original volume. Samples of both the supernatant and the pellet are dried in test tubes and 10 ml of concentrated H_2SO_4 added. These preparations are assayed for PHBA according to the methods of Slepecky and Law (1960), and for total carbohydrate using the anthrone procedure.

8. Proteins were routinely measured using the method of Warburg and Christian (1941). Difference before and after TCA extraction was used as the measure of unhydrolyzed protein.

9. When precise measurements of protein content were desired, the colorimetric method of Lowry, et al. (1951) was used. Bovine Serum Albumin was used as the standard.

10. Total amino acids were measured by the spectrophotometric method of Warburg and Christian (1941). Proteins were first precipitated out of solution using 5% TCA.

11. Amino acids were also measured by determining the tyrosine and tryptophan content using a modification of the colorimetric method of Spiess (1957). To 1.0 ml of a solution containing amino acids was added 5.0 ml of 0.1 N NaOH containing 2% Na_2CO_3; 0.5 ml of Folin-Ciocalteau Reagent (Harleco) diluted in water to roughly 1 N acidity

was immediately added, and the solution was mixed. After 40 minutes at room temperature, the optical density of the solution was read at 660 nm. The intensity of the blue color developed by this method is proportional to the concentration of tyrosine and tryptophan in solution up to at least 160 g tyrosine/ml. Tyrosine was used as a standard and had a molar extinction coefficient of 2.15×10^3; that of tryptophan should be the same.

In solutions containing protein as well as amino acids, the protein was precipitated with addition of trichloroacetic acid to a 5% final concentration. The precipitate was removed after 30 minutes at 23°C, and the amino acids in the supernatant liquid were measured as described above. Trichloroacetic acid does not interfere with the colorimetric test, but as it absorbs strongly in the ultraviolet, rigorous quantitative care must be employed when estimating amino acids spectrophotometrically in its presence.

12. Nucleotides were determined by the method of Warburg and Christian (1941), provided that ratio, O.D.$_{280\ nm}$: O.D.$_{260\ nm}$, was greater than 0.7.

13. Dry weight of sludge samples was determined gravimetrically after drying the sludge to constant weight at 105°C. The sludge solids were not washed before drying, and the reported values are high by virtue of soluble matter in the sludge liquid. However, as washing decreases the weight of the samples by only 1-2%, it was considered an unnecessary operation.

B. SUPPLEMENTARY STUDIES

PHYSICAL FACTORS AFFECTING SLUDGE DEFLOCCULATION

Biological Dewatering in the Presence of Gels

Gum, acacia, gelatin, cellulose gum, agar, Methocel HG, all at 0.1% level, 0.01% fresh egg albumen, and the thick, sticky capsular growth of a previously isolated bacterium (SL-30), were added separately to one liter of fresh sludge and the sedimentation rates timed. The first experiment involving gelatin appeared rather promising, in that gelatin seemed to increase the rate of sedimentation during the first half of the observation period, and it was on this basis that additional experiments were conducted (Table B-1). However, subsequent experiments employing gelatin did not substantiate that the addition of this material appreciably affected the settling rate of sludge. The daily fluctuation of the composition of sludge would appear to be involved, in that the nature of the sludge may allow it to be slightly affected positively by an agent, such as gelatin, on one day, and unaffected by the same agent on a second day.

This type of experiment would appear to be worth further pursuit, i.e., testing more products of biological origin - for example, other bacterial capsular materials - for their effects on the sedimentation rate of sludge.

TABLE B-1

Sedimentation Rates Of Sludge Containing Biological Polymers

Whittier Narrows sludge containing 1.0% of the indicated gel agent except for egg albumin at 0.1% was placed in 11 graduated cylinders. The height of the sludge was read as it settled upon standing undisturbed.

Time	Gum Acacia	Gelatin	Cellulose Gum	Control
0	1000 ml	1000 ml	1000 ml	1000 ml
10 min	940 ml	810 ml	980 ml	900 ml
20 min	890 ml	660 ml	960 ml	760 ml
30 min	830 ml	570 ml	940 ml	600 ml
40 min	775 ml	520 ml	910 ml	500 ml
50 min	710 ml	480 ml	880 ml	430 ml
60 min	640 ml	450 ml	850 ml	390 ml

Time	Gelatin	Egg Albumen	Methocel	Control
0	1000 ml	1000 ml	1000 ml	1000 ml
10 min	990 ml	980 ml	980 ml	1000 ml
20 min	985 ml	970 ml	970 ml	990 ml
30 min	980 ml	960 ml	960 ml	980 ml
40 min	970 ml	960 ml	955 ml	970 ml
50 min	970 ml	955 ml	950 ml	970 ml
60 min	970 ml	950 ml	940 ml	950 ml

Time	Gelatin	SL-30	Agar	Control
0	1000 ml	1000 ml	1000 ml	1000 ml
10 min	1000 ml	990 ml	1000 ml	995 ml
20 min	990 ml	975 ml	990 ml	990 ml
30 min	980 ml	970 ml	980 ml	980 ml
40 min	970 ml	960 ml	970 ml	970 ml
50 min	960 ml	950 ml	960 ml	960 ml
60 min	955 ml	940 ml	950 ml	960 ml

Deflocculation Studies

Heat: Calleja (1970) was able to deflocculate the yeast *Schizosaccharomyces pombe* by means of heat, papain, and 5 M urea. By extension of his work, use of the general number of free bacteria viewed in a microscopic preparation as an index of deflocculation appeared possible. That is, it was assumed that the greater number of bacteria seen outside of floccules shortly after a given treatment, the greater the amount of deflocculation that had taken place as a result of the treatment. Using the above concept as the basis for experimentation, fresh sludge was centrifuged at low speed (1000 rpm for 3 min) so that any bacteria already existing free in the sludge were left suspended in the supernatant fluid and subsequently discarded during the sludge washing procedure. The sludge was washed three times at low speed with, and resuspended in, deionized water.

One hundred ml of the resuspended sludge were placed in a water bath and heated at 80°C for 3 hrs. During this time, microscopic preparations were made and examined in the phase microscope. By the end of 1 hour of heating, there were definitely more free bacteria than existed in the starting material, the heated fluid seemed to be more viscid, and the sludge had changed color from brown to grey. Whether heating beyond an hour's duration increased the number of bacteria that were freed from the floccules was difficult to decide.

Urea: Concentrated solutions of urea have the ability to break hydrogen bonds in polymeric substances and accordingly testing the effect of urea on sludge was thought worthwhile. Urea was added to fresh sludge that had been water washed three times to give a final concentration of 5M urea (the pH remained at 6.8). This mixture was left at (about 22°C) and it was monitored at intervals by examination in the phase microscope. Again, freed bacteria seen in the microscope were the criterion that the floccule had been attacked and reduced in structural complexity. However, relatively few free bacteria were in fact seen, even at the end of three hours of exposure to urea, and the floccules appeared to be unchanged.

Heat and Urea: The next step was to combine any action of both heat and urea. This was accomplished by heating to 80°C thrice-washed sludge in which was dissolved 5 moles of urea. In 15 min a fairly large number of free bacteria could be seen in the microscope, although the floccules looked normal. After the heated urea-sludge mixture had been allowed to settle, a very turbid supernate was obtained over a brown sediment, the supernatant yielding many freed bacteria, and the sediment showing granular appearing floccules, perhaps indicative of dissolution of some of the matrix.

Effects of Aeration on Dewatering

Air

Three and a half liters of fresh Whittier Narrows sludge were diluted 1:1 with tap water in order to maintain a high dissolved oxygen level and aerated by means of a compressed air line for 5-1/2 months; the volume was kept constant by the daily addition of water. Samples were compared with fresh WN sludge diluted 1:1.

Periodically, samples of the aerated sludge were used experimentally to spread on agar plates containing activated sludge, as an inoculum for various other media, and for irradiation with ultraviolet light in attempts to get out organisms that are able to lyse floc matrix material. At 3 weeks of aeration, sedimentation tests, employing 100 ml of the aerated sludge, showed the phenomenon where the sludge at the bottom of the graduated cylinder settled out to a volume of about 8 ml whereas the turbidity of the overlying sludge remained the same at the 100 ml mark. At 5-1/2 months of aeration, settling of the sludge did not occur even after several hours. The initial pH of the sludge was 6.8; it dropped to 4.5 three or four days after the start of aeration and remained at this level until the experiment was terminated.

The solids content of the diluted sludge fell 15% after 9 weeks of aeration and was about 13% below the starting weight at the end of the 5-1/2 month period. Organisms showing some lysis on sludge agar plates were plentiful after 5-1/2 months of aeration, but no organisms showing exceptional lytic properties were obtained.

Oxygen

Studies were made of the introduction of pure oxygen into sludge to overcome a potential deficiency of dissolved oxygen, thereby invigorating sludge organisms and allowing them to reinitiate active metabolism, possibly involving matrix dissolution.

Two experiments were carried out in which 400-500 ml of fresh sludge at room temperature were oxygenated for 6 hours with pure oxygen through bubbling and compared with a similar volume of sludge aerated by bubbling with compressed air. Oxygenation of the sludge was further aided by means of a magnetic stirrer. Two grams of NH_4Cl were added to the oxygenated sludge to provide a source of nitrogen for the bacteria, in that work previously had shown nitrogen to be lacking in the sludge supernatant.

Subsequent filtration studies (Table B-2) showed that the oxygenated sludge filtered more slowly than the aerated sludge, even after supernatant replacement. Examination in the microscope indicated that the oxygenated sludge contained more free bacteria than did the aerated sludge. Oxygenation for longer periods of time may have given more positive results where dewatering is concerned.

TABLE B-2

Effects of Oxygenation on Filtration Rates

At room temp., 400 ml of fresh sludge was aerated by means of a compressed air line. 2g. of NH_4Cl were added to a companion flask and pure oxygen was bubbled through the sludge. The oxygenated flask was placed on a magnetic stirrer to increase the oxygenation. Filtration tests were made at 6 and at 20 hours.

Sludge Treatment	θ/V^2	θ/V^2 Air / θ/V^2 Oxygen	Supernatants Replaced	
			θ/V^2	θ/V^2 Air / θ/V^2 Oxygen
At 6 Hours				
Air	66.7	1.0	28.6	1.0
Oxygen + NH_4Cl	133.3	0.5	22.2	1.3
At 20 Hours				
Air	50.0	1.0	28.6	1.0
Oxygen + NH_4Cl	66.6	0.75	40.0	0.7

DETERMINATION OF NUCLEIC ACIDS IN MATRIX EXTRACTS

After methanol precipitation of centrifuged alkaline sludge supernatant, dry weights were determined on samples of the material after resuspension in deionized water. A sample of the precipitate was then dialyzed overnight against several changes of deionized water and dry weight samples were again obtained. RNase and DNase were then added to the dialyzed material and this was again dialyzed. Weight determinations indicated that about 50% of the starting material had been lost as a result of these treatments.

Ultraviolet absorption spectra of the initial alkaline extract material revealed an absorption peak at 258 nm apparently indicating the presence of some nucleic acids. There was still some UV-absorbing material in this spectral region after dialysis and treatment with the nucleases. These materials also gave positive responses to ninhydrin reagent after paper chromatographic separation.

The sonicated extract, when examined in the ultraviolet-spectrophotometer, revealed a flat peak in the region of 240-265 nm; the methanol-precipitated material from this sonicate was dissolved in deionized water and this gave a curve of the same general shape as for the alkaline extract.

The cysteine-sulphuric acid reaction (Colowick and Kaplan, 1957) for the detection of deoxyribonucleic acid (DNA) was performed on extracts obtained from sludge by various means, e.g., alkaline extraction, hot-water extraction, etc., in order to determine if nucleic acids were being extracted simultaneously from sludge at the time that matrix material was being extracted. Color reactions of various kinds (pink, orange, brownish) were obtained with all samples tested, although these color reactions do not necessarily mean that DNA was involved in that other compounds may give color responses (DNA gives a pink color with a peak at 490 nm that appears with maximum intensity after 10 to 15 min). UV-absorption curves of this type were found in hot-water extracts from 70° and 80°C and blended acetone-extracted sludge. Filtration through activated charcoal (which removes nucleotides) left a small peak at 255 nm when alkaline extract was so treated (inorganic phosphate, sugar phosphates, acetyl phosphate, salts, and proteins are not adsorbed to charcoal in significant amounts). Visible-light spectral analysis of the cysteine-sulphuric acid reaction of alkaline extract revealed a peak at 485 nm, which is very close to the 490 nm expected of DNA, allowing for spectral shifts due to the salt concentrations, etc. Small contaminating amounts of nucleic acids can be concluded to be present in the extracted materials.

Some preliminary experiments were carried out on the acetone extract of blended sludge using the Schneider hot, dilute trichloracetic acid (TCA) method (Colowick and Kaplan, 1957) for the extraction of nucleic acids. In the TCA extract, the shape of the absorption curve normally seen in the alkaline extracted material was flattened out in the 260-280 nm region, indicating that very little nucleic acid was present and no protein.

SLUDGE SUPPLEMENTATION EXPERIMENTS

Autoclaved Sludge as a Selective Medium

An important objective was to isolate microorganisms that could utilize sludge matrix as a nutrient. Since it was evident that normal viable sludge greatly resists the establishment of competing organisms, autoclaved sludge was used as the culture medium. Observations had been made earlier that autoclaved sludge agar serves as a culture medium for a variety of microorganisms. It was fully recognized that autoclaved sludge might well be changed in its properties from unautoclaved native sludge, so that organisms that may be able to attack the former medium may not be able to attack the latter.

Flasks containing autoclaved sludge were inoculated with a variety of complex sources of microorganisms, including field soils and horse dung. The flasks were incubated at room temperature on a shaker and examined daily for a period of 28 days. It was appreciated that a considerable time might be required before a matrix-attacking organism(s) could establish itself and reveal its activities. Eventually, three passages of these materials were made in autoclaved sludge; sludge agar plates were spread from the flasks at intervals as a rough index of the (level) of "lytic" organisms contained in the flasks.

A number of organisms grew in the autoclaved sludge and were carried through the long-term passages. However, viewed microscopically, the sludge floccules remained unchanged throughout the period of observation, and the number of "lytic" organisms did not increase noticeably or change their obvious characteristics.

Growth of Microbial Isolates on Autoclaved Sludge

In a somewhat similar manner to the above, a "lytic" organism, obtained through the various techniques already described, was inoculated into autoclaved sludge. Second passages were made, although once again there were no indications of changes in the floccules.

Filtration tests were conducted on the above cultures. The growth supernatants, and therewith any sludge attacking enzymes present, were added to normal sludge sediment. The mixture was filtered after the growth supernates were given time (75 min) to act upon the normal sludge floc.

The filtration rates of the experimental samples were greatly slower than those of the normal sludge control. Boiled growth supernatant fluids (to inactivate any enzymes present), which were added to normal sludge floc, filtered at rates similar to unboiled supernatants. Filtration rates of representative "lytic" cultures in autoclaved sludge made at 18 hr. did not differ significantly from those made at 46 hr., thereby indicating that sludge-attacking enzymes did not appear in either young or old cultures.

Addition of Nutrients to Sludge

One may argue logically that the reason the sludge matrix material, as demonstrated in the preceding section, is not broken down is because the majority of the bacteria in the floccules are either dead, dormant, moribund, or senescent, and hence unable to form the enzyme(s) necessary to attack the matrix. These bacteria are in this state because they lack nutrients that are no longer supplied by the sludge itself. By this reasoning, if external nutrients are supplied directly to normal sludge, the bacteria may be revivified with concomitant enzymatic activities, some of which might be directed toward the dissolution of the sludge floccules.

Whole Sludge Supplementation

Fresh sludge was diluted 1:5 with tap water in order to ensure that aeration of the sludge-containing flasks would be as complete as possible when they were placed on a shaker.

Organic nitrogen (0.1 to 1%) was supplied in the following forms - peptone, tryptone, beef heart infusion, peptonized milk, casamino acids, and glycine; ammonium chloride served as an inorganic nitrogen source. Yeast extract (0.001%) was used to provide growth factors. Carbon (0.1 to 1%) sources included alcohols (methanol, ethanol, propanol, butanol), formic acid, sodium acetate, and carbohydrates (glucose, sucrose, soluble starch, maltose, and lactose).

The above substances were either added alone to 100 ml of diluted sludge, or added together in various combinations, such as peptone plus yeast extract, etc.

Dewatering results are typified by those illustrated in Table B-3 where values are presented for some of the earliest attempts at supernatant replacement studies. In none of the supplemented sludge experiments did filtration rates show marked improvement over those of the normal sludge controls when tested in the routine manner.

Solids Phase Supplementation

These studies developed directly from the nutrient addition experiments. In these investigations, normal sludge was regarded as being composed of both a solid and liquid media, neither of which (alone or together) was adequate to support microorganisms whose enzymatic repertoire might include the ability to attack floccules. The purpose of these studies was to add supplements either to washed sludge or to sludge supernatant fluid and to examine the media for their ability to promote active microbial growth.

Fresh WN sludge was centrifuged and washed three times with tap water. It was then used (1) as an inoculum in Pseudomonas basal medium, where the sludge itself acted as both the carbohydrate source

TABLE B-3

Effects of Nutrient Supplementation on Filtration Rates

Supplement Added to Autoclaved Sludge	Supernatant from normal Sludge		Supernatant from Microbial Isolates	
	θ/V^2	Ratio $\frac{\theta/V^2 \text{ control}}{\theta/V^2 \text{ supplement}}$	θ/V^2	Ratio $\frac{\theta/V^2 \text{ control}}{\theta/V^2 \text{ supplement}}$
Normal Sludge Control	7.00	1.00	8.80	1.00
0.05% methanol	6.60	1.06	16.00	0.56
0.05% ethanol	7.50	0.93	18.70	0.47
0.05% propanol	8.40	0.83	17.00	0.51
0.05% butanol	12.50	0.56	10.00	0.88
0.05% formic acid	18.00	0.39	16.60	0.53
0.05% glycine	12.00	0.59	7.70	1.14
0.05% peptone	5.60	1.25	8.00	1.10

for the basal medium and also as the bacterial inoculum, or (2) suspended in tap water as a basal medium itself to which was added various supplements; here it also served as either its own bacterial inoculum or a field soil was added as the inoculum. Repeated passages in the same medium followed in attempted enrichment culture procedures. Flasks were aerated on a gyrotary shaker at room temperature.

The supplements included various combinations of peptone (0.01%), yeast extract (0.001%), glucose (0.01%), and a mixed salt solution containing NaCl, K_2HPO_4, $MgSO_4$, $CaCl_2$, $FeSO_4$, and $MnCl_2$.

After three passages in the Pseudomonas medium there appeared to be some effect on the floccules with time, but this was not unequivocal.

After 18 hr of incubation of the first passage, washed sludge in (1) mixed salts + yeast extract + peptone, and (2) the same ingredients + glucose, showed some floccules that appeared to have lost some of their matrix material. Two more passages in the same media did not seem to produce any further changes in the floccules. There were no obvious changes in the passages involving the soil inocula.

Representative "lytic" cultures were inoculated on to a medium in which the water washed and resuspended solid phase (matrix) of sludge was solidifed with 1.5% agar. These organisms grew well and produced cleared areas around their colonies which were, if anything, slightly larger than those on ordinary, unwashed sludge agar plates, almost as if some inhibitory substance(s) had been removed through washing.

Liquid Phase Supplementation

Corollary experiments to the above, employing normal sludge supernatant as a growth medium, supplemented with materials to supply organic nitrogen (peptone), carbon and energy (glucose), and growth factors (yeast extract), in various combinations, were set up as indicated below:

1. Supernatant alone (as a control)
2. Supernatant + 0.005% yeast extract
3. Supernatant + 0.005% peptone
4. Supernatant + 0.005% glucose
5. Supernatant + yeast extract + peptone + glucose
6. Autoclaved supernatant alone + SL-1 culture
7. Autoclaved supernatant + yeast extract + peptone + glucose + SL-1

8. Autoclaved supernatant + SL-33 culture

9. Autoclaved supernatant + yeast extract + peptone + glucose + SL-33.

Flasks 1 to 5 were inoculated with one loopful of the centrifuged sludge pellet, flasks 6 and 7 with the "lytic" bacteria culture SL-1, and flasks 8 and 9 with the "lytic" bacteria culture SL-33. All flasks were incubated at room temperature on the gyrotary shaker.

Samples from the above flasks were removed and examined at intervals under the phase contrast microscope. Growth developed fastest in flasks 5, 7, and 9. Growth did not occur in flask 6, indicating that autoclaved sludge supernatant did not contain nutrients sufficient to allow the development of SL-1. By 12 days of incubation, the nutrients in the supplemented supernatant were exhausted, as evidenced by the absence of bacterial cells in most of the flasks when viewed in the microscope.

Thus it is seen that sludge supernatant fluid can support the growth of a variety of bacteria if it is supplemented, at least minimally, with other nutrient sources.

Attempts to Obtain "Lytic" Mutants by Ultra-Violet Irradiation

Irradiation of "Lytic" Organisms

Because a number of microorganisms from a variety of sources had been found that produced "halos" around their colonies on sludge agar plates, it was thought worthwhile to expose a number of these organisms to ultra-violet irradiation. Ultra-violet irradiation is a recognized mutagen that might induce mutations that would exhibit this "lytic" phenomenon to a much greater degree. Accordingly, "lytic" organisms were diluted in sterile water, 0.1 ml of each dilution was plated on sludge agar plates, and the plates exposed at a distance of 30 cm to the radiation from a gemicidal U.V. tube for periods from 1 sec to 10 min., with due precautions to prevent photo reactivation.

These plates were then incubated at room temperature (22°C), 30°C, and 37°C, since variation in the incubation temperatures should allow maximal opportunity for the selection of mutants.

Also, it was known that the optimal growth temperature of "lytic" organisms was not always the best temperature for demonstrating the lytic phenomenon. For example, at the end of six days of incubation, culture SL-11 had shown best growth and lysis at 37°C; SL-19 - best lysis at room temperature and best growth at 37°C; SL-23 - best growth at 30°C and best lysis at room temperature.

None of the U.V. survivors of this procedure produced zones of lysis around their colonies on sludge agar plates that exceeded those of the organisms prior to irradiation.

Irradiation of Diluted Sludge

It was considered possible to obtain sludge lytic microorganisms directly from diluted sludge that had been exposed to ultra-violet irradiation.

Sludge was diluted $1{:}10^7$ in sterile tap water; 0.1 ml was spread on each of 3 sludge agar plates that were then exposed to U.V. light for periods from 5 sec to 1 min. Whereas a few "lytic" colonies were detected after incubation, none of these showed any extraordinary lytic zones that warranted further study.

Irradiation of Known Bacillus Cultures

Because a number of the "lytic" organisms that had been randomly isolated were aerobic, Gram-positive, and spore-forming, which are the characteristics of the genus Bacillus, known cultures of *Bacilli*, namely, *Bacillus cereus* and *B. megaterium*, were inoculated into Nutrient Broth and subsequently spread on sludge agar plates and exposed to U.V. for 1-5 min. Plates were incubated at room temperature (22°C), 30°C, and 37°C.

It was already known that these *Bacillus* cultures prior to irradiation caused zones of "lysis" around their colonies on sludge agar plates and it was hoped that survival from irradiation might bring with it increased enhancement of the lytic phenomenon. Unfortunately, such was not the case.

Since the large number of "lytic" organisms, which have been isolated during the course of these studies, uniformly grow well in nutrient broth and on nutrient agar, experiments have included irradiation of "lytic" organisms spread on nutrient agar plates. The thought behind such experiments was that "super" lytic organisms might possibly survive initially on nutrient agar which would not survive irradiation on sludge agar. However, subculture of nutrient agar survivors onto sludge agar as an indicator of "super" lysis did not reveal any enhanced lytic ability.

ATP Supplementation of Sludge

Leakage of amino acids may be due to a deficiency of energy metabolism reflected in loss of integrity of the cell membrane.

One could interpret the continued existence of sludge matrix material to mean that sludge bacteria in their dormant or senescent state do not have enough energy available in the form of energy-rich bonds, i.e., ATP (adenosine 5' - triphosphate) in order to initiate the metabolic pathway of matrix degradation. By this reasoning, the addition of external ATP could make up for this energy deficiency and allow the bacteria to attack the matrix as a carbohydrate source. Furthermore, inorganic nitrogen, added to eliminate the possibility of a nitrogen nutritional deficiency, should promote and enhance the metabolic activity of the sludge bacteria. The assumption is made here that the ionic requirements for growth, cofactors, etc., would be met by the sludge itself.

In order to test these hypotheses, flasks containing 100 ml of fresh WN sludge were treated as follows and incubated on the gyrotary shaker:

1) Sludge control

2) Sludge + 0.1 gm ATP

3) Sludge + 0.1 gm ATP + 0.5 gm NH_4Cl.

Many free bacteria were seen microscopically in flask (3) at 18 hrs. Filtration was more rapid from the control sludge than from either of the ATP-containing samples, although after supernatant replacement, the initially slower filtering ATP + NH_4Cl, now slightly exceeded the ATP sample (Table B-4).

It would appear that the addition of ATP, even in substantial quantity, was not the trigger with which to start the metabolic attack on the matrix material by the sludge organisms. Many suggestions could be advanced to explain the lack of response, such as, for example, that the organisms were too old or moribund to respond to the addition of the ATP.

Molds Displaying Lytic Properties

Because mold cultures produce a large number of exoenzymes simultaneously during growth, it was decided to test mold fluids for matrix-attacking enzymes. Four different mold cultures were inoculated onto sludge-moistened wheat bran that had been either autoclaved before inoculation or left unautoclaved. The molds grew very slowly and, at times varying from 12 to 20 days, the cultures were extracted with 100 ml of 0.02 M phosphate buffer (pH 7.0) and the liquids centrifuged to free them from the molds. Uninoculated bran was similarly treated as a control.

To test for the presence of any sludge attacking enzymes, fresh Whittier Narrows sludge was centrifuged in 25 ml amounts and the supernatant fluid saved. Ten ml of each of the different mold extract fluids were then added to the sludge pellets which were resuspended in the mold fluids and left at room temperature for 90 min. in order to allow time for any enzymatic activity to take place.

TABLE B-4

ATP Supplementation of Sludge

0.1 g ATP (adenosine 5' - triphosphate-Sigma) was added to each of two flasks containing fresh sludge. To one flask was also added 0.5 g NH_4Cl. Flasks were incubated at room temp. on a gyrotary shaker.

Sludge Treatment	θ/V^2	$\frac{\theta/V^2 \text{ Control}}{\theta/V^2 \text{ Treated}}$	Supernatants Replaced θ/V^2	Supernatants Replaced $\frac{\theta/V^2 \text{ Control}}{\theta/V^2 \text{ Treated}}$
Control	50	1.0	11.7	1.0
+ ATP	75	0.7	22.2	0.5
+ ATP + NH_4Cl	100	0.5	16.6	0.7

At the end of this time, the sludge samples were centrifuged again and the original sludge supernatant was used to resuspend the sludge pellets. The sludge was then filtered in the standard way.

None of the mold-extracted fluids passed through the filter paper as rapidly as the control fluid. This lack of obvious enzymatic activity may have been a result of poor timing, viz., younger mold cultures may have contained active enzymes of value that the older cultures did not.

ENZYME SCREENING PROGRAM

Filtration Rate of Enzyme Treated Sludge at Different pH Values

In each of these experiments fresh sludge was centrifuged and resuspended to roughly 5% (w/v) solids in a small amount of the supernatant liquid, in order to increase the concentration of any possible enzyme substrates. The concentrated sludge was incubated with various commercial enzymes at 400μg protein/ml. After 60 minutes at 23°C, the reaction mixtures were diluted with buffers at various pH values, including that in the original reaction mixture. The filtration rates of the mixtures were compared with those of appropriate controls from which enzyme had been omitted.

As shown in Tables B-5, B-6, B-7, and B-8, samples of fresh sludge incubated with α-amylase (with or without added Mg^{++}), β-amylase, cellulase, chitinase, DNAase, lipase, lysozyme, peptidase, pronase, RNAase, or trypsin show at every pH value tested a similar rate of filtration to the reaction mixtures from which enzyme had been omitted.

In these experiments the filterability varied with pH as would be predicted from the results reported previously in which the most rapid dewatering rate occurred at pH 3. The values of the ratio, filtration rate without enzyme/filtration rate with enzyme, show some minor fluctuations probably due to difficulties inherent in the methods. No significant improvements (5 to 10X) in filterability was observed.

Filtration Rate of Enzyme-Treated Sludge After 24-hour Incubation

The foregoing experiments were done with a relatively short incubation time of one hour. Since certain of the enzyme substrates, particularly cellulose, are often hydrolyzed rather slowly, it was desirable to extend the period of incubation. The experiments were therefore repeated. The sludge samples were incubated at 23°C for 24 hours in the presence or absence of the different enzymes, and the filtration rates were again compared. As observed before, the enzymes had no significant effect on the filterability of sludge. These experiments are summarized in Tables B-9, B-10, B-11, and B-12.

TABLE B-5

Dewatering of enzyme-treated sludge at different pH values

Fresh sludge was concentrated to 3.2% (w/v) solids by centrifugation. Reaction mixtures contained 6.5 ml of concentrated sludge, 0.75 ml of 0.2M pH 7 phosphate buffer, or 0.75 ml 0.2M pH 4.8 citric acid-citrate buffer. 0.3 ml of enzyme at 10 mg protein/ml, or 0.3 ml H_2O were then added, and the mixtures were incubated at 23°. After one hour, the mixtures were diluted with (1) 15.1 ml of 0.1M pH 7.0 phosphate buffer; (2) 15.1 ml 0.1M pH 5.0 citrate buffer; or (3) 15.1 ml 0.1M pH 3.0 citrate buffer. The filtration and sedimentation rates of these diluted mixtures were measured.

A Additions to reaction mixture	B pH		C	D[(a)]
	During incubation	During filtration	θ/V^2	$\frac{\theta/V^2 \text{ in } H_2O}{\theta/V^2 \text{ in enzyme}}$
H_2O	7.0	7.0	128	--
H_2O	7.0	5.0	375	--
H_2O	7.0	3.0	52	--
Lipase	7.0	7.0	128	1.00
Lipase	7.0	5.0	190	1.96
Lipase	7.0	3.0	62	0.84
Trypsin	7.0	7.0	128	1.00
Trypsin	7.0	5.0	585	0.64
Trypsin	7.0	3.0	50	1.03
Cellulase	4.8	7.0	128	1.00
Cellulase	4.8	5.0	375	1.00
Cellulase	4.8	3.0	72	0.73

[(a)] Determined from the rates in Column C at comparable pH values of filtration.

TABLE B-6

Dewatering of enzyme-treated sludge at different pH values

Fresh sludge was concentrated to 4.02% (w/v) solids by centrifugation. Reaction mixtures contained 6.5 ml of concentrated sludge and 0.75 ml of 0.2M pH 7 phosphate buffer. 0.3 ml of enzyme at 10 mg protein/ml, or 0.3 ml H_2O, were then added, and the mixtures were incubated at 23°. After one hour, the mixtures were diluted with (1) 15.1 ml of 0.1M pH 7.0 phosphate buffer; (2) 15.1 ml 0.1M pH 5.0 citrate buffer; or (3) 15.1 ml 0.1M pH 3.0 citrate buffer. The filtration and sedimentation rates of these diluted mixtures were measured.

A Additions to reaction mixture	B pH		C	D[(a)]
	During incubation	During filtration	θ/V^2	$\dfrac{\theta/V^2 \text{ in } H_2O}{\theta/V^2 \text{ in enzyme}}$
H_2O	7.0	7.0	152	--
H_2O	7.0	5.0	400	--
H_2O	7.0	3.0	70	--
Pronase	7.0	7.0	156	1.00
Pronase	7.0	5.0	400	1.00
Pronase	7.0	3.0	220	0.32
α amylase	7.0	7.0	156	1.00
α amylase	7.0	5.0	400	1.00
α amylase	7.0	3.0	85	0.83
Chitinase	7.0	7.0	158	1.00
Chitinase	7.0	5.0	400	1.00
Chitinase	7.0	5.0	90	0.78

(a) Determined from the rates in column C at comparable pH values of filtration.

TABLE B-7

Dewatering of enzyme-treated sludge at different pH values

Fresh sludge was concentrated to 4.3% (w/v) solids by centrifugation. Reaction mixtures contained 6.5 ml of concentrated sludge and 0.75 ml of 0.2M pH 7 phosphate buffer. 0.3 ml of enzyme at 10 mg protein/ml, or 0.3 ml H_2O, were then added, and the mixtures were incubated at 23°. After one hour, the mixtures were diluted with (1) 15.1 ml of 0.1M pH 9.0 Tris-HCl; (2) 15.1 ml of 0.1M pH 7.0 phosphate buffer; (3) 15.1 ml of 0.1M pH 5.0 acetate buffer; or (4) 15.1 ml of 0.1M pH 3.6 acetate buffer. The filtration rates of these diluted mixtures were measured.

A Additions to reaction mixture	B pH During incubation	 During filtration	C θ/V^2	D[a] $\frac{\theta/V^2 \text{ in } H_2O}{\theta/V^2 \text{ in enzyme}}$
H_2O	7.0	9.0	300	--
H_2O	7.0	7.0	290	--
H_2O	7.0	5.0	200	--
H_2O	7.0	3.6	160	--
β amylase	7.0	9.0	395	0.75
β amylase	7.0	7.0	400	0.73
β amylase	7.0	5.0	160	1.25
β amylase	7.0	3.6	155	1.07
RNAase	7.0	9.0	290	1.03
RNAase	7.0	7.0	245	1.18
RNAase	7.0	5.0	100	2.00
RNAase	7.0	3.6	115	1.39
DNAase	7.0	9.0	290	1.03
DNAase	7.0	7.0	165	1.76
DNAase	7.0	5.0	300	0.67
DNAase	7.0	3.6	95	1.68

(a) Determined from the rates in column C at comparable pH values of filtration.

TABLE B-8

Dewatering of enzyme-treated sludge at different pH values

Fresh sludge was concentrated to 3.57% (w/v) solids by centrifugation. Reaction mixtures contained 6.5 ml of concentrated sludge and 0.75 ml of 0.2M pH 7 phosphate buffer. $MgCl_2$, at a final concentration of $10^{-3}M$, was added to the reaction mixture containing α-amylase to enhance enzymatic activity.

Reaction mixtures were incubated 60' at 23°. At this time reaction mixtures were diluted with (1) 15.1 ml of 0.1M pH 7.0 phosphate buffer; (2) 15.1 ml of 0.1M pH 9.0 Tris-HCl; (3) 15.1 ml of 0.1M pH 5.0 acetate buffer; or (4) 15.1 ml of 0.1M pH 3.6 acetate buffer. The filtration rates of these diluted mixtures were measured.

A Additions to reaction mixture	B		C	D(a)
	During incubation	During filtration	θ/V^2	$\frac{\theta/V^2 \text{ in } H_2O}{\theta/V^2 \text{ in enzyme}}$
H_2O	7.0	9.0	340	--
H_2O	7.0	7.0	200	--
H_2O	7.0	5.0	145	--
H_2O	7.0	3.6	115	--
Lysozyme	7.0	9.0	195	1.74
Lysozyme	7.0	7.0	190	1.05
Lysozyme	7.0	5.0	120	1.21
Lysozyme	7.0	3.6	130	0.88
α amylase, $MgCl_2$	7.0	9.0	180	1.88
"	7.0	7.0	350	0.57
"	7.0	5.0	210	0.69
"	7.0	3.6	136	0.85
Peptidase	7.0	9.0	260	1.31
Peptidase	7.0	7.0	500	0.40
Peptidase	7.0	5.0	230	0.63
Peptidase	7.0	3.6	130	0.88

(a) Determined from the rates in Col. C at comparable pH values of filtration.

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TABLE B-9

Dewatering of Enzyme Treated Sludge at Different pH Values after 24 Hours Incubation

Fresh sludge was concentrated to 3.2% (w/v) solids by centrifugation. Reaction mixtures contained 40 ml of sludge and 4.6 ml of 0.2 M pH 7 phosphate buffer. 1.85 ml of enzyme at 10 mg protein/ml or 1.85 ml H_2O were then added and the mixtures incubated at 27°C. After 24 hours, 2 ml of each mixture was diluted with (1) 8.0 ml 0.2 M pH 9.0 Tris buffer; (2) 8.0 ml 0.2 M pH 7.0 phosphate buffer; (3) 8.0 ml 0.2 M pH 5.0 acetate buffer; or (4) 8.0 ml pH 3.6 acetate buffer.

A Additions to reaction mixture	B pH		C	D[(a)]
	During incubation	During filtration	θ/V^2	$\frac{\theta/V^2 \text{ in } H_2O}{\theta/V^2 \text{ in enzyme}}$
H_2O	7.0	9.0	72	--
H_2O	7.0	7.0	164	--
H_2O	7.0	5.0	46	--
H_2O	7.0	3.6	24	--
PHBA depolymerase	7.0	9.0	72	1.0
PHBA "	7.0	7.0	92	1.74
PHBA "	7.0	5.0	40	1.15
PHBA "	7.0	3.6	29	1.00
Trypsin	7.0	9.0	72	1.00
Trypsin	7.0	7.0	108	1.51
Trypsin	7.0	5.0	44	1.04
Trypsin	7.0	3.6	39	0.83
Cellulase	7.0	9.0	72	1.00
Cellulase	7.0	7.0	120	1.36
Cellulase	7.0	5.0	38	1.21
Cellulase	7.0	3.6	21	1.14

(a) Determined from the rates in column C at comparable pH values of filtration.

TABLE B-10

Dewatering of Enzyme Treated Sludge at Different pH Values after 24 Hours Incubation

Fresh sludge was concentrated to 3.2% (w/v) solids by centrifugation. Reaction mixtures contained 40 ml of sludge and 4.6 ml of 0.2 M pH 7 phosphate buffer. 1.85 ml of enzyme at 10 mg protein/ml or 1.85 ml H_2O were then added and the mixtures incubated at 27°C. After 24 hrs, 2 ml of each mixture was diluted with (1) 8.0 ml 0.2 M pH 9.0 Tris buffer; (2) 8.0 ml 0.2 M pH 7.0 phosphate buffer; (3) 8.0 ml 0.2 M pH 5.0 acetate buffer; or (4) 8.0 ml pH 3.6 acetate buffer.

A Additions to Reaction Mixture	B pH During incubation	 During filtration	C θ/V^2	D[a] θ/V^2 in H_2O / θ/V^2 in enzyme
H_2O	7.0	9.0	230	-
H_2O	7.0	7.0	196	-
H_2O	7.0	5.0	155	-
H_2O	7.0	3.6	78	-
β-amylase + $MgCl_2$	7.0	9.0	185	1.24
β-amylase + $MgCl_2$	7.0	7.0	230	0.85
β-amylase + $MgCl_2$	7.0	5.0	110	1.41
β-amylase + $MgCl_2$	7.0	3.6	62	1.25
Lysozyme	7.0	9.0	315	0.73
Lysozyme	7.0	7.0	200	0.98
Lysozyme	7.0	5.0	92	1.69
Lysozyme	7.0	3.6	60	1.30
Lipase	7.0	9.0	119	1.94
Lipase	7.0	7.0	255	0.77
Lipase	7.0	5.0	102	1.52
Lipase	7.0	3.6	44	1.77

[a] Determined from the rates in Column C at comparable pH values of filtration.

TABLE B-11

Dewatering of Enzyme Treated Sludge at Different pH Values after 24 Hours Incubation

Fresh sludge was concentrated to 3.2% (w/v) solids by centrifugation. Reaction mixtures contained 40 ml of sludge and 4.6 ml of 0.2 M pH 7 phosphate buffer. 1.85 ml of enzyme at 10 mg protein/ml or 1.85 ml H_2O were then added and the mixtures incubated at 27°C. After 24 hours, 2 ml of each mixture was diluted with (1) 8.0 ml 0.2 M pH 9.0 Tris buffer; (2) 8.0 ml 0.2 M pH 7.0 phosphate buffer; (3) 8.0 ml 0.2 M pH 5.0 acetate buffer; or (4) 8.0 ml pH 3.6 acetate buffer.

A Additions to reaction mixture	B pH		C	D[a]
	During incubation	During filtration	θ/V^2	$\frac{\theta/V^2 \text{ in } H_2O}{\theta/V^2 \text{ in enzyme}}$
H_2O	7.0	9.0	141	--
H_2O	7.0	7.0	118	--
H_2O	7.0	5.0	82	--
H_2O	7.0	3.6	37	--
RNAase	7.0	9.0	400	0.35
RNAase	7.0	7.0	122	0.97
RNAase	7.0	5.0	123	0.67
RNAase	7.0	3.6	56	0.66
DNAase	7.0	9.0	184	0.77
DNAase	7.0	7.0	300	0.39
DNAase	7.0	5.0	81	1.01
DNAase	7.0	3.6	44	0.84
α-Amylase	7.0	9.0	248	0.57
α-Amylase	7.0	7.0	130	0.91
α-Amylase	7.0	5.0	122	0.67
α-Amylase	7.0	3.6	72	0.52

(a) Determined from the rates in column C at comparable pH values of filtration.

TABLE B-12

Dewatering of Enzyme Treated Sludge at Different pH Values after 24 Hours Incubation

Fresh sludge was concentrated to 3.2% (w/v) solids by centrifugation. Reaction mixtures contained 40 ml of sludge and 4.6 ml of 0.2 M pH 7 phosphate buffer. 1.85 ml of enzyme at 10 mg protein/ml or 1.85 ml H_2O were then added and the mixtures incubated at 27°C. After 24 hours, 2 ml of each mixture was diluted with (1) 8.0 ml 0.2 M pH 9.0 Tris buffer; (2) 8.0 ml 0.2 M pH 7.0 phosphate buffer; (3) 8.0 ml 0.2 M pH 5.0 acetate buffer; or (4) 8.0 ml pH 3.6 acetate buffer.

A Additions to reaction mixture	B pH		C θ/V^2	D[a] $\frac{\theta/V^2 \text{ in } H_2O}{\theta/V^2 \text{ in enzyme}}$
	During incubation	During filtration		
H_2O	7.0	9.0	98	--
H_2O	7.0	7.0	80	--
H_2O	7.0	5.0	62	--
H_2O	7.0	3.6	48	--
Papain	7.0	9.0	100	0.98
Papain	7.0	7.0	88	0.91
Papain	7.0	5.0	61	1.01
Papain	7.0	3.6	67	0.72
Pronase	7.0	9.0	92	1.06
Pronase	7.0	7.0	80	1.00
Pronase	7.0	5.0	60	1.03
Pronase	7.0	3.6	43	1.11
Peptidase	7.0	9.0	172	0.57
Peptidase	7.0	7.0	336	0.24
Peptidase	7.0	5.0	88	0.71
Peptidase	7.0	3.6	152	0.32

(a) Determined from the rates in Column C at comparable pH values of filtration.

The commercially available enzymes, pectinase, hyaluronidase, acylase, neuraminidase, collagenase, elastase, and β-glucuronidase were also examined under various conditions of pH and incubation intervals for their effects on dewatering.

Tables B-13 through B-19 present the θ/V^2 values obtained for these enzymes, as well as a comparison of the ratio, filtration rate without enzyme (water control) filtration rate with enzyme.

With one exception, the filtration rates of sludge appeared to be unchanged by incubation with this group of enzymes. Acylase did appear, from the tables, to exert some effect on the dewatering rates. However when the experiment was repeated the enhanced dewatering effect was not evidenced.

TABLE B-13

Dewatering of Pectinase-Treated Sludge at Different Solids Concentration

Fresh sludge was concentrated to 3.0% - 3.4% solids (w/v) by centrifugation. Reaction Mixtures contained 6.5 ml of concentrated sludge and 0.75 ml of 0.1 M pH 4 citrate buffer; 0.3 ml of enzyme at 10 mg protein/ml, or 0.3 ml H_2O, was then added, and the mixtures were incubated at 23°C. After 1 or 24 hrs the mixtures were diluted with 15.1 ml of 0.1 M pH 7.0 phosphate buffer. The filtration rates of the diluted mixtures were then measured.

Addition to Reaction Mixture	% Solids	Incubation Time (hrs)	pH		θ/V^2	$\frac{\theta/V^2 \text{ in } H_2O}{\theta/V^2 \text{ in enzyme}}$
			During Incubation	During Filtration		
H_2O	3.06	1	4	7	75	-
H_2O	3.06	24	4	7	54	-
H_2O	3.14	1	4	7	45	-
H_2O	3.14	24	4	7	62	-
H_2O	3.40	1	4	7	84	-
H_2O	3.40	24	4	7	52	-
Pectinase	3.06	1	4	7	68	1.10
Pectinase	3.06	1	4	7	62	1.21
Pectinase	3.06	24	4	7	64	0.84
Pectinase	3.06	24	4	7	76	0.71
Pectinase	3.14	1	4	7	58	0.78
Pectinase	3.14	1	4	7	86	0.52
Pectinase	3.14	24	4	7	68	0.91
Pectinase	3.14	24	4	7	62	1.00
Pectinase	3.14	24	4	7	58	1.07

TABLE B-14

Dewatering of Pectinase-Treated Sludge at Different pH Values

Fresh sludge was concentrated to 3.4% solids (w/v) by centrifugation. Reaction mixtures contained 6.5 ml concentrated sludge and 0.75 ml of (1) 0.1 M pH 4 citrate buffer, (2) 0.1 M pH 5.4 citrate buffer, or (3) 0.1 M pH 7.0 phosphate buffer. 0.3 ml of pectinase at 10 mg protein/ml, or 0.3 ml H_2O, was then added, and the mixtures were incubated at 23°C. After 1 or 24 hrs, the mixtures were diluted with 15.1 ml of 0.1 M pH 7.0 phosphate buffer, and the filtration rates of the diluted mixtures were measured.

Addition to Reaction Mixture	Incubation Time (hrs)	pH		θ/V^2	θ/V^2 in H_2O
		During Incubation	During Filtration		θ/V^2 in enzyme
H_2O	1	7.0	7	84	-
H_2O	24	4.0	7	52	-
Pectinase	1	4.0	7	66	1.27
Pectinase	1	5.4	7	66	1.27
Pectinase	1	7.0	7	42	2.00
Pectinase	24	4.0	7	46	1.13
Pectinase	24	5.4	7	62	0.84
Pectinase	24	7.0	7	46	1.13

TABLE B-15

Dewatering of Hyaluronidase-Treated Sludge at Different pH Values

Fresh sludge was concentrated to 2.74% solids (w/v) by centrifugation. Reaction mixtures contained 6.5 ml of concentrated sludge and 0.75 ml of (1) 0.1 M pH 4 citrate buffer, (2) 0.1 M pH 5.4 citrate buffer, or (3) 0.1 M pH 7 phosphate buffer. 0.3 ml of hyaluronidase at 10 mg protein/ml, or 0.3 ml H_2O, was then added, and the mixtures incubated at 23°C. After 2 or 24 hours, the mixtures were diluted with 15.1 ml of 0.1 M pH 7 phosphate buffer, and the filtration rates were then measured.

Addition to Reaction Mixture	Incubation Time (hrs)	pH		θ/V^2	$\frac{\theta/V^2 \text{ in } H_2O}{\theta/V^2 \text{ in enzyme}}$
		During Incubation	During Filtration		
H_2O	2	7.0	7	54	-
H_2O	24	7.0	7	54	-
Hyaluronidase	2	4.0	7	58	0.93
Hyaluronidase	2	5.4	7	50	1.00
Hyaluronidase	2	7.0	7	68	0.79
Hyaluronidase	24	4.0	7	82	0.66
Hyaluronidase	24	5.4	7	78	0.69
Hyaluronidase	24	7.0	7	58	0.93

TABLE B-16

Dewatering of Collagenase, Elastase, and β-Glucuronidase-Treated Sludge

Addition to Reaction Mixture	Sludge % Solids	Incubation Time (hrs)	pH		θ/V^2	$\frac{\theta/V^2 \text{ in } H_2O}{\theta/V^2 \text{ in enzyme}}$
			During Incubation	During Filtration		
H_2O	2.94	1	7.0	7	41	-
H_2O	2.94	24	7.0	7	56	-
Collagenase	2.94	1	7.0	7	47	0.87
Collagenase	2.94	24	7.0	7	60	0.93
H_2O	2.70	1	8.8	7	64	-
H_2O	2.70	24	8.8	7	72	-
Elastase	2.70	1	8.8	7	78	0.82
Elastase	2.70	24	8.8	7	79	0.91
H_2O	2.79	1	5.0	7	90	-
H_2O	2.79	24	5.0	7	155	-
β-glucuronidase	2.79	1	5.0	7	91	0.99
β-glucuronidase	2.79	24	5.0	7	180	0.86

TABLE B-17

Dewatering of Acylase-Treated Sludge at Different pH Values

Fresh sludge was concentrated to 2.74% solids (w/v) by centrifugation. Reaction mixtures contained 6.5 ml of concentrated sludge and 0.75 ml of (1) 0.1 M pH 5.4 citrate buffer, (2) 0.1 M pH phosphate buffer, or (3) 0.1 M pH 8 phosphate buffer. 0.3 ml of acylase at 10 mg protein/ml, or 0.3 ml of H_2O, was added, and the mixtures were then incubated at 23°C. After 1 or 24 hours, the mixtures were diluted with 15.1 ml of 0.1 M pH 7 phosphate buffer, and the filtration rates of the diluted mixtures were measured.

Addition to Reaction Mixture	Incubation Time (hrs)	pH		θ/V^2	θ/V^2 in H_2O / θ/V^2 in enzyme
		During Incubation	During Filtration		
H_2O	1	7.0	7	39	-
H_2O	24	7.0	7	78	-
Acylase	1	5.4	7	62	0.63
Acylase	1	7.0	7	48	0.81
Acylase	1	8.0	7	40	1.02
Acylase	24	5.4	7	38	2.05
Acylase	24	7.0	7	38	2.05
Acylase	24	8.0	7	38	2.05

TABLE B-18

Dewatering of Acylase-Treated Sludge at Alkaline pH Values

Fresh sludge was concentrated to 3.12% solids (w/v) by centrifugation. Reaction mixtures contained 6.5 ml of concentrated sludge and 0.75 ml of (1) 0.1 M pH 7.9 Tris-HCl buffer, (2) 0.1 M pH 9.1 Tris-HCl buffer, or (3) 0.1 M pH 10.2 Tris-HCl buffer. 0.3 ml acylase at 10 mg protein/ml, or 0.3 ml H_2O, was then added, and the mixtures were incubated at 23°C. After 1 or 24 hrs the mixtures were diluted with 15.1 ml of 0.1 M pH 7.9 Tris-HCl buffer, and the filtration rates of the diluted mixtures were measured.

Addition to Reaction Mixture	Incubation Time (hrs)	pH		θ/V^2	$\frac{\theta/V^2 \text{ in } H_2O}{\theta/V^2 \text{ in enzyme}}$
		During Incubation	During Filtration		
H_2O	1	7.9	7.9	82	-
H_2O	24	7.9	7.9	74	-
Acylase	1	7.9	7.9	194	0.42
Acylase	1	9.1	7.9	94	0.87
Acylase	1	10.2	7.9	100	0.82
Acylase	24	7.9	7.9	144	0.51
Acylase	24	9.1	7.9	90	0.82
Acylase	24	10.2	7.9	58	1.28

TABLE B-19

Dewatering of Acylase and Neuraminidase-Treated Sludge

Addition to Reaction Mixture	Sludge % Solids	Incubation Time (hrs)	pH		θ/V^2	θ/V^2 in H_2O
			During Incubation	During Filtration		θ/V^2 in enzyme
H_2O	2.76	1	7.0	7.0	80	-
H_2O	2.76	24	7.0	7.0	68	-
Acylase	2.76	1	5.4	7.0	94	0.85
Acylase	2.76	1	7.0	7.0	84	0.95
Acylase	2.76	1	8.0	7.0	64	1.25
Acylase	2.76	24	5.4	7.0	76	0.84
Acylase	2.76	24	7.0	7.0	72	0.94
H_2O	2.61	1	7.2	7.2	51	-
H_2O	2.61	24	7.2	7.2	46	-
Neuraminidase	2.61	1	7.2	7.2	45	1.13
Neuraminidase	2.61	24	7.2	7.2	40	1.15
H_2O	2.68	2	7.2	7.2	106	-
Neuraminidase + Acylase	2.68	2	7.2	7.2	106	1.00

1 *Accession Number*

2 *Subject Field & Group*

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5 *Organization*

Envirogenics Co.
Division of Aerojet-General Corporation
El Monte, California

6 *Title*

Biological Methods of Sludge Dewatering

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*Sludge Treatment, *Dewatering, *Enzymes, Filtration, Oxygen Requirements, Amino Acids, Sewage Bacteria

25 *Identifiers (Starred First)*

Sludge Matrix, Vacuum Filters

27 *Abstract*

Improved dewatering of sewage sludge has been accomplished for the first time by biological means; namely, through the agency of the enzyme lysozyme acting under controlled conditions. Improved dewatering was measured by a 4 to 8-fold increase in filtration rate, which, if extrapolated to plant scale operations, should result, in a given time period, in a 2 to 3-fold increase in solids yield, thus achieving a significant cost saving. A number of other exo- and endo-enzymes were also tested for their effects on dewatering, with uncertain results. Several microorganisms were isolated that showed lysis around their colonies when they were grown on agar plates containing sludge. Attempts were made to enhance this lytic property through the isolation of ultraviolet resistant mutants that exhibited the phenomenon to an increased degree. Selection of matrix-solubilizing organisms was attempted by means of enrichment culture procedures that used an alkaline extract of sludge as the selective medium, which allows the removal of filter-clogging materials that would otherwise obscure the positive action of sludge modifying enzymes. Of major significance is the demonstration that the relative mild conditions of a biological process permit attacking the sludge dewatering process at its source - the matrix material. (Mechalas - Envirogenics Co.)

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